MINIREVIEW

Manipulating Corynebacteria, from Individual Genes to Chromosomes

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Corynebacterium glutamicum is an industrial organism with a long history of use for the production of various fine chemicals. The *C. glutamicum*-mediated production of L-glutamic acid by fermentation was one of the very first industrial processes of the biotechnology era. This fermentation originated in Japan from the discovery in 1957 by Kinoshita et al. (cited by Kumagai [68]) that, under suitable conditions, this soil bacterium is able to secrete L-glutamic acid in significant amounts (66). This process successfully replaced less cost-effective methods based on hydrolysis by concentrated hydrochloric acid of soy or various other plant proteins. Glutamic acid was traditionally extracted from seaweed to serve as a seasoning agent and remains to this day a very important food flavor additive, the worldwide production of which approximates 1.5 million tons per year (43). Industrial glutamic acid fermentation was implemented early on by major Japanese companies, thus building over time exquisite manufacturing knowledge at various industrial scales up to approximately 5,000 hectoliters (43). In the 1970s, efficient *C. glutamicum* L-lysine and L-threonine producers were generated by random mutagenesis and positively selected by phenotypic resistance to lysine or threonine analogs. The advent of molecular biology enabled a new wave of development in which this industrial know-how was leveraged, not only to improve the performance of the existing lysine and threonine production processes, but also to enable the production of other amino acids and vitamins (29, 43, 51, 68). The utilization of recombinant DNA techniques, combined with metabolic and carbon flux analyses (67, 99), facilitated the identification of metabolic bottlenecks and their bypassing by expressing or repressing the corresponding genes to develop further improved amino acid industrial production processes. The intrinsic characteristics of this food grade microbial workhorse include its lack of pathogenicity and its lack of spore-forming ability, both desirable traits as listed by the U.S. Center for Biologics Evaluation and Research and the U.S. Center for Drug Evaluation and Research guidelines, as well as its high growth rate, its relatively limited growth requirements, the ability of several strains not to undergo autolysis under conditions of repressed cell division (50), the absence of native extracellular protease secretion that makes corynebacteria suitable hosts for protein expression (10, 100), and the relative stability of the corynebacterial genome itself (85). These intrinsic attributes, combined with an up-to-date

set of genetic-engineering tools, make this organism ideal for the development of robust industrial processes that are increasingly competitive compared to *Escherichia coli*-, *Bacillus subtilis*-, or yeast-based processes. As a result, corynebacterial fermentations have become increasingly relevant to a wide range of industrial sectors, including food, feed, cosmetic, pharmaceutical, and chemical companies.

Nonetheless, the significance of the ability to manipulate corynebacteria by genetic engineering is not limited to industrial considerations, as corynebacteria also constitute ideal models for understanding the biology of other genera from the same monophyletic taxon, including *Dietzia*, *Gordonia*, *Mycobacterium*, *Nocardia*, *Skermania*, *Tsukamurella*, *Turicella*, and *Williamsia* species (72, 74, 98, 111). Moreover, in addition to the human pathogen *Corynebacterium diphtheriae*, which is still responsible for epidemics associated with significant mortality and morbidity (113), corynebacteria encompass a variety of ill-understood pathogenic, commensal, and opportunistic pathogenic organisms (33), as exemplified by the observation that the resident flora of the hands, which constitutes an important host defense mechanism against invasion by pathogenic species, mostly comprises coryneform bacteria and coagulase-negative staphylococci (11, 77).

The completion of the *C. glutamicum* genome sequence (48, 60) marks the dawn of a new era in corynebacterial research in which genomewide genetic manipulations that have the potential to generate more efficient and more versatile whole-cell industrial biocatalysts become possible. The purpose of this review is to revisit the various recombinant DNA tools that are available to implement this aim, with an emphasis on *C. glutamicum*, and to define the next major objectives to be accomplished in this area.

TRANSFORMATION TECHNIQUES

Given the lack of any apparent natural or chemically induced DNA uptake system akin to the traditional methods of transformation of *E. coli* or *B. subtilis*, practical improvement of corynebacteria stemmed from the development of fast, easy, and reproducible transformation techniques. From the early successes of protoplast or spheroplast techniques as a means to circumvent the extracellular membrane barrier to conjugation or transduction methods (reviewed by Kirchner and Tauch [63]), DNA transfer techniques in corynebacteria came of age with the successes attained in the 1990s with electrotransformation. The basic principle of electroporation is to create transient pores in the bacterial cell wall where a variety of extracellular molecules can gain access to a cell's cytosol

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(132, 143). In the presence of transforming DNA, this phenomenon leads either to transient or to stable expression of the genes thus taken up, depending on whether the foreign DNA molecules can circumvent the restriction barrier and whether they can replicate. It is now well established that the dielectric breakdown of the cell's outer boundaries is aided by weakening of the cell wall induced by growth conditions. For example, we reported that growing *C. glutamicum* cells in the presence of penicillin G, a known inhibitor of cell wall synthesis, increased the transformation efficiency by approximately 2 orders of magnitude compared with the control (69). Other cell wall-weakening methods include the addition of ampicillin (15) or glycine, which weaken the cross-linking of the peptidoglycan chains (40). Similarly, the addition of isonicotinic acid hydrazide and Tween 80 was proven to be beneficial (42). Isonicotinic acid hydrazide decreases the mean length of the mycolic acid carbon chains, and Tween 80 is known to alter the composition of the mycolic acid layer (19, 112). The physiological function of the corynebacterial mycolic acid layer typically present in the bacteria of the *Corynebacterium-Mycobacterium-Nocardia* taxonomic cluster is primarily to serve as a second permeability barrier surrounding the peptidoglycan layer and to confer resistance to chemical injury (7). A detailed protocol on *C. glutamicum* transformation, as well as a number of corynebacterial physiological and practical aspects, has recently been published (28).

In addition to the physical barrier constituted by the cell wall, the biological barrier enforced by the *mrr*- and *mcr*-like restriction and modification system of corynebacteria must also be overcome (139). A variety of practical methods have been developed to reach efficiencies of up to approximately 5×10^7 transformants per μg of DNA, such as isolating the transforming DNA from *dam* or *dcm* *E. coli* modification mutants (139) and other suitable heterologous sources (12), using synthetic DNA (2) or restriction-deficient mutants (16, 73), applying a heat shock treatment (133), or modifying the transforming DNA by the action of the *C. glutamicum* methyltransferase (102, 125). On the other hand, it is noteworthy that the technique of intergeneric protoplast fusion has been successfully applied in corynebacteria (23, 135), where, as observed in other bacterial and fungal systems (37, 79, 94), it generated progeny with improved properties that resulted from chromosomal recombination rather than through complementing heterozygote formation.

VECTORS

Stemming from the isolation of various endogenous plasmids (24, 127), numerous shuttle and cloning vectors have been designed to manipulate corynebacteria. These have been extensively reviewed elsewhere (see, for example, Kirchner and Tauch [63], Martin and Gil, [76], Nakata et al. [86], and Srivastava and Deb [110]). Several heterologous antibiotic resistance genes have been demonstrated to be useful positive selection markers in corynebacteria, such as genes conferring resistance to chloramphenicol, kanamycin, bleomycin, erythromycin, spectinomycin, and gentamicin (63, 86). On the other hand, a few antibacterial resistance determinants have been isolated from *C. glutamicum* and closely related genera, includ-

ing genes conferring resistance to chloramphenicol, erythromycin, kanamycin, streptomycin-spectinomycin, tetracycline, trimethoprim, and sulfonamides (reviewed by Kirchner and Tauch [63] and Tauch et al. [124, 127]). It is also worth noting that auxotrophic complementation schemes represent an interesting alternative (69, 121) as a means to industrialize corynebacterial production strains and to facilitate compliance with the guidelines of governmental bodies, such as the Center for Biologics Evaluation and Research or the Center for Drug Evaluation and Research, that regulate the biotechnology industry in the United States.

Native *C. glutamicum* plasmids have been classified into four distinct families based on their putative modes of replication and the degree of amino acid sequence similarity of their RepA proteins (127). While very efficient cloning vectors, such as the pCRB series, have been developed (86), it may be advantageous to base a larger number of industrial expression plasmids on native episomes that replicate via a theta mechanism, such as pXZ10142 or pCRY4 (127), rather than via a rolling-circle mode, as the latter mode of replication has been associated in *B. subtilis* with segregational and structural instability (30, 58). Nevertheless, segregational stability can be reinforced by the cloning of *trans*- or *cis*-acting regions favoring plasmid maintenance (70, 84, 134).

Expression issues have been mostly solved using several *E. coli* promoters that have been observed to remain functional in corynebacteria (82). A recent study demonstrated that numerous promoters of *C. glutamicum* exhibit a functional structure similar to that of common bacterial promoters, with extensive similarity existing between the -10 hexamers of *C. glutamicum* and *E. coli* promoters (93). For example, the P_{tac} promoter (22, 106) remains a strong promoter in *C. glutamicum*. Likewise, a variety of corynebacterial promoters have been isolated by means of promoter-probe vectors in order to enable controlled gene expression in these bacteria and have been demonstrated to remain functional in *E. coli* (5, 17, 31, 144). Transcription terminators have been identified using a similar strategy (5, 110). Nonetheless, several *C. glutamicum* promoters appear not to be functional in *E. coli*, such as the promoters of the *thrA* and *lysA* genes (reviewed by Srivastava and Deb [110]). Likewise, the phage P-104 promoter and the promoters of the *hom* and the *leuA* genes do not function in *B. subtilis* (92). Perhaps the most significant observation for the genetic engineering of *C. glutamicum* is that the technology developed to manipulate *E. coli* or *B. subtilis* is to a large extent easily transferable to corynebacteria. However, repressible and inducible gene expression systems still need to be tailored to optimize gene expression in industrial applications of coryneform bacteria, for example, by implementing simple and cost-effective control systems that respond to temperature shifts, as exemplified by Delaunay et al. (26), or to the addition during the fermentation of an inexpensive reactant. This industrial objective requires more fundamental research effort to understand the fine biology of the signals of gene transcription and translation in corynebacteria, as well as the mechanisms modulating protein activity in these bacteria, such as activator-repressor couples, sensing two-component cascades, and cross talk phenomena between various such systems.

ISOLATION OF NATIVE MOBILE GENETIC ELEMENTS

The isolation of corynebacterial mobile genetic elements, which are mostly cryptic in nature, was made possible by the development of positive entrapment procedures based on the observation that overexpression of the *B. subtilis* levansucrase (25, 71) in the presence of large amounts of sucrose is toxic to coryneform bacteria (57), as it is to gram-negative bacteria (35). This strategy was successfully applied to clone, for example, the insertion sequence IS31831 from *C. glutamicum* (138). This insertion sequence was subsequently demonstrated to spontaneously transpose at a high frequency when its host cells are subjected to various stresses, such as inhibitor challenge (34) or heat shock treatments (6). A number of other insertion sequences have also been isolated, but belonging to a relatively low number of different mobile-element families, as defined by Mahillon and Chandler (75). Insertion sequences from the ISL3 family that have been isolated from corynebacteria include IS31831 and its isoforms, named ISCg1 (56) and IS1207 (13), as well as IS13869, an insertion sequence that is 79% identical to IS31831 at the amino acid level (20). Moreover, two additional functional IS31831 isoforms were isolated as part of transposon Tn14751, a 20.3-kb composite transposon native to *C. glutamicum*. Tn14751 comprises several open reading frames, including purine biosynthesis genes but neither antibiotic resistance nor antibiotic synthesis genes (52). The presence of mobile elements from the IS3 family, which is characterized by two consecutive and partially overlapping open reading frames, was observed in the chromosome of *C. glutamicum* (IS1206) (14) and on native plasmids of *Corynebacterium jeikeium* (119). IS14999, isolated from *C. glutamicum* (131), represents the only member of the IS630/Tc1 mobile element family identified to date in corynebacteria. This particular mobile genetic element is more closely related to eukaryotic Tc1/*mariner* elements, known not to require any host factor for transposition, than to prokaryotic elements. Likewise, ISCg2 (IS30 family), which exhibits a pronounced target specificity (95); IS6100 (IS6 family), recovered from the polyantibiotic resistance plasmid pTET3 (120); and IS1249 (IS256 family), which forms transposon Tn5432 from the 51-kb *Corynebacterium striatum* polyantibiotic resistance plasmid pTP10 (126), are also to date the only representatives of their respective families. It is worth noting that the increased knowledge of the biology of corynebacteria isolated from clinical samples (33), as well as the sequences of whole corynebacterial genomes (18, 48, 60, 87, 122), resulted in a significant enrichment of available useful plasmids, transposons, and insertion sequences, such as IS3504 from the *Corynebacterium diphtheriae* plasmid pNG2 (118) or Tn5564 from plasmid pTP10 (126).

TRANSPOSON MUTAGENESIS

The ideal attributes of transposable elements as genetic tools include active transposition into the target organism, randomness of integration sites, high integration frequency, stability of the transposition mutants, a controllable number of insertion events per transformation, compatibility with other mobile genetic elements, and absence in the chromosome of the target organism. On the other hand, widely distributed mobile genetic elements form the basis of epidemiological

studies and strain-typing methods (96). Transposition activity can be engineered either by genetic engineering or by biochemical approaches. Genetic-engineering approaches rely, for example, on increasing the activity of the transposase or on modifying native regulatory frameshifts. On the other hand, biochemical approaches rely, for instance, on introducing by electroporation a transposome prepared in vitro that contains the transposase protein bound to the segment of DNA to be integrated, which comprises at a minimum the transposon ends and a positive selection marker (38). Likewise, the number of transposition events per transformation and the stability of the resulting transposition mutants can both be modulated using a minitransposon derived from a mobile element which is absent from the target organism (i.e., the transposase is provided in *cis* or in *trans* of the mobile element). However, randomness of integration and compatibility with other genetic elements are both best ensured by using systems based on native elements that intrinsically exhibit these characteristics.

Among the most useful transposition mutagenesis systems constructed for coryneform bacteria are those based on IS31831 (136) and its isoform isolates (13, 109). This insertion sequence is included, for example, in miniTn31831 and Tn5531, respectively. For example, we have routinely achieved efficiencies of approximately 4×10^4 transposon mutants per μg DNA at essentially random locations, as demonstrated by the observation that 0.2% of these mutants exhibit various auxotrophic phenotypes, though the integration sites are typically AT rich. This observation is consistent with results attained with those other members of the ISL3 family that form a tight phylogenetic cluster with IS31831 (e.g., IS13869, IS1096, and ISBli1) (52). Similar observations were made using a kanamycin-resistant minitransposon derivative of transposon Tn14751. This recombinant element was shown to integrate into the genome of *C. glutamicum* at an efficiency of 1.8×10^2 transformants per μg of DNA (52). Tn14751 thus has the potential not only to serve as a mutagenic agent, but also to serve as a delivery vehicle of large DNA fragments to the corynebacterial genome. Notably, these systems are complemented by Tn5-derived technology, since the gram-negative transposon Tn5, which does not require any host factor for transposition, has been shown to integrate at essentially random locations in various hosts, including *Corynebacterium matruchotii* (117) and *C. diphtheriae*, where efficiencies of transposition of 2×10^5 transposon mutants per μg of DNA have been attained (89). We have used a combination of these two systems to achieve global transposon mutagenesis and generated, from a pool of 13,000 mutants, a library of 2,300 different *C. glutamicum* transposon mutants covering 75% of the *C. glutamicum* genome. Of the remaining 25%, 131 open reading frames show homology to genes that have been demonstrated to be essential in *E. coli*. In contrast, we attained transposition mutation in 39 genes homologous to other essential *E. coli* genes (N. Suzuki, N. Okai, H. Nonaka, Y. Tsuge, M. Inui, and H. Yukawa, poster 15, Abstr. Int. Workshop Biorefinery, Kyoto, Japan, 2005). In addition, the erythromycin resistance IS1249 composite transposon Tn5432 from the IS256 family of mobile elements, and isolated from plasmid pTP10, was also demonstrated to be a useful tool. In particular, it was shown to integrate relatively randomly, as suggested by the observation that 0.2% of the isolated mutants are auxo-

trophs of different natures but in sites containing a triple A/T sequence. This element nevertheless has the particularity to generate cointegrate at a frequency of 98% (123, 124). Moreover, the chloramphenicol resistance transposon Tn5564, likewise originating from plasmid pTP10, was demonstrated, using a conjugation/mobilization system, to be capable of transposing in *C. glutamicum* at a conjugation frequency of 3.3×10^{-8} (128). This transposon inserts into target sites containing the 4-bp palindrome CTAG. Similarly, the 27.8-kb *C. glutamicum* antibiotic resistance plasmid pTET3 harbors insertion sequences capable of transposition in *C. glutamicum*. Among these, IS6100 was shown to be transposable by conjugation at a frequency of 2.8×10^{-6} (120). Results reported to date promote the view that IS6100 integrates in a relatively random fashion (120).

GENE DISRUPTION AND REPLACEMENT

Chromosomal gene disruption and replacement methods for corynebacteria were first developed using a conjugation/mobilization system of *E. coli* vectors (107). Efficient host/vector-independent integrative transformation was subsequently achieved by electroporation when the corynebacterial restriction system was successfully circumvented, for example, by using synthetic DNA or DNA passaged in a suitable corynebacterial host (45, 97) or by using nonmethylated plasmid DNA (137). We observed that integration of nonreplicative plasmids occurs at an efficiency of up to approximately 10^2 integrants per μg of DNA and typically results at 98% from Campbell-like integration events and at 2% from double-crossover events. As a complementary technique to transposon mutagenesis, this method is automatable using a scheme where DNA coding for the desired sites of integration can be generated by PCR or subcloned in a library format, ligated in vitro to a suitable positive selection marker, and used to transform *C. glutamicum*, preferably by conjugation and mobilization, since to date only a limited number of electroporation samples can be treated simultaneously. We used this strategy successfully to generate random mutants of *C. glutamicum* from a library of genomic Sau3A DNA fragments used as regions of homology with the chromosome. This process is largely facilitated by the applicability of the *B. subtilis* *sacB* gene as a conditionally lethal marker (57) to positively select the rare mutants that result from a double-crossover event. The *sacB* gene also proved useful to design markerless methods of gene disruption and replacement in corynebacteria (49). Furthermore, convenient multipurpose mobilizable vectors, such as pK18mobsacB, have been constructed to enable defined deletions in *C. glutamicum* (103). It is also noteworthy that various tools for site-specific integration based on the presence in multiple copies of specific chromosomal sequences, such as the insertion sequence IS13869 or the 16S rRNA genes, were designed to increase the number of integrated copies of genes of interest (reviewed by Kirchner and Tauch [63]). On the other hand, vectors based on corynephages represent performing tools for site-specific integration of useful sequences into the *C. glutamicum* genome (80, 81).

GENOME SEQUENCE

To date, at least six corynebacterial genomes have been entirely sequenced: two different isolates of *C. glutamicum* strain ATCC 13032 (48, 60) and strain R (H. Nonaka, P. Kós, N. Okai, M. Inui, and H. Yukawa, poster 9, Abstr. Int. Workshop Biorefinery, Kyoto, Japan, 2005), *Corynebacterium efficiens* YS-314 (87), *C. jeikeium* K411 (122), and *C. diphtheriae* NCTC 13129 (18). A comparative study of the *Corynebacterium* genome was reported by Kalinowski (59), who observed by analyzing the genomes of *C. glutamicum*, *C. efficiens*, and *C. diphtheriae* a common core of approximately 1,600 genes. Similarly, 52% (1,089) of the open reading frames identified in the *C. jeikeium* genome are orthologous with genes from these three species, thus promoting the view that this subset of genes constitutes the conserved corynebacterial chromosomal backbone (122). Moreover, the conservation of the gene order among these microbes is unusually high. This observation could perhaps be ascribed to the lack of a RecBCD system encoding the DNA recombinational repair system in these bacteria, the absence of which could have prevented gene shuffling and thus maintained the gene order that existed in these species prior to their divergence (85). The genome of the human pathogen *C. diphtheriae* and that of the human nosocomial pathogen *C. jeikeium*, both approximately 2.5 Mb in size, are the smallest of those of the four genera fully sequenced to date. The *C. glutamicum* R genome is 3,314,179 bp long. We compared this sequence with that of the published 3,309,401-bp *C. glutamicum* ATCC 13032 genome (48). We estimated that strain R, with a G+C content of 54.1%, comprises approximately 3,000 genes, similar to the observation made by Ikeda and Nakagawa (48) and Kalinowski et al. (60), who independently annotated the 53.8% G+C content *C. glutamicum* ATCC 13032 genome. Nevertheless, an unambiguous determination of the exact number of genes present in *C. glutamicum* would require an in-depth examination of all the putative open reading frames of unknown functionality that are present in the organism. We identified a total of 11 species-specific DNA islands greater than 10 kb in size in the genome of *C. glutamicum* strain R. In addition, the largest of the strain ATCC 13032-specific DNA islands is 218 kb in size and corresponds to a low-G+C-content region of the ATCC 13032 genome which contains putative prophage sequences. This observation confirms the alien origin of this segment, which was either acquired by horizontal transfer by strain ATCC 13032 or lost by strain R after their divergence. These species-specific sequences may form the basis of typing and taxonomic methods that may be useful to determine the lineages and the evolution of various corynebacterial strains. On the other hand, the availability of annotated complete genomic sequences of corynebacterial strains enables one to develop more predictive models of cellular behavior. Beyond the immediate goal of designing production strains bearing only useful mutations and no deleterious mutations, as would inevitably derive from a classical chemical mutagenesis and selection scheme (88), the full genome sequence brings about a genuine change of engineering scale: from genes to chromosomes. Both physiology and genetics can now be addressed in a global fashion. Physiology studies have been facilitated by the subsequent development in DNA arrays and proteomics that allow

global gene expression analysis (67) and protein identification on a scale not possible before (90). Transcription profiling, combined with detailed studies of intracellular metabolite concentrations and the definition of metabolic fluxes, proved to be instrumental in determining the key metabolic properties of, for example, a lysine production strain (67). On the other hand, genetic manipulation can now be envisaged at the megabase level.

DNA ARRAYS

DNA arrays constitute tools that enable one to conduct multiplex analyses of various aspects of physiology and regulation phenomena and thus generate important economies of scale and scope that ultimately result in the acceleration of the pace of research. Arrays comprising virtually all of the predicted *C. glutamicum* genes have been prepared by several groups (46, 83). A simpler version of the open reading frame-based array is the shotgun DNA array, in which a library of genomic fragments covering the genome several times is immobilized on a solid support. These fragments can harbor one or more open reading frames or encode only intergenic regions. These particular arrays have been found to be potent tools to select genes being regulated under certain conditions without prior cost-intensive sequencing effort. However, in contrast to the transcription profiles obtained using open reading frame-based arrays, data resulting from the use of such arrays do not reflect relative transcript abundance. The ability to determine the variation of mRNA levels under a variety of experimental conditions remains important in deciphering, for example, global regulation mechanisms of carbon, energy, nitrogen, and phosphorous metabolism (140). Beyond transcription profiling, DNA arrays are practical tools for genotyping to detect differences that exist between two strains at the nucleotide level (8, 61, 140). As such, they are useful in identifying the mutations obtained by classical mutagenesis methods and thus the transferring of these mutations to engineer a minimal mutation strain that harbors only those mutations that have a positive impact on the targeted application (88). Furthermore, the understanding of the molecular bases of beneficial mutations is a key to unraveling the fundamental mechanisms affected, and thus to enabling the design of more efficient production strains, for instance, by the implementation of global metabolic-engineering techniques.

CHROMOSOME MANIPULATION

To fully benefit from the fundamental knowledge now being gained at a pace faster than ever, one needs to be able to elevate the genetic-engineering scale from that of the individual gene to the megabase level. Natural evolution teaches us that bacterial genomes have a certain inherent plasticity, where megabase deletions, duplications, inversions, and insertions, as well as the presence of megabase-size plasmids, have played a major role in the makeup of present-day organisms (130). An example of the plasticity of the microbial genome is provided by the artificial dissection of the *B. subtilis* genome into two stably maintained replicons 3,878 kb and 310 kb in size (55). Likewise, Anagnostopoulos (1) reported genetic rearrangement in a *B. subtilis* strain, the chromosome of which increased

in size by several hundred kilobases following a megabase duplication event. We have developed a Cre/loxP-mediated system for large genome rearrangements in *C. glutamicum* enabling iterative and independent deletions (114–116). We applied this technique to delete 11 strain-specific DNA islands in the genome of *C. glutamicum* R representing 250 kb, or 7.5% of the genome. As expected, despite the loss of several putative open reading frames, the mutant cells exhibited normal growth patterns under standard laboratory conditions, though no competition experiments with wild-type strains were conducted. The deletion of these strain-specific islands represents a first step in the reduction of the genome size in an attempt to streamline physiological pathways, as cells contain numerous genes that only marginally contribute to cellular fitness and various dispensable cycles that are not essential for survival (78, 129), particularly when cells are grown in rich media. Similarly, the feasibility of integrating large heterologous DNA fragments has been demonstrated in bacteria by integration into the chromosome of *B. subtilis* of a mouse genomic DNA segment of approximately 120 kb by way of the ordered assembly of 20-kb to 50-kb murine DNA fragments (54). Transposon systems, such as Tn14751 (52), or site-specific integration systems based on corynebacteriophage (80, 81), constitute potential delivery vehicles for megabase insertion of DNA sequences into the genome of corynebacteria.

POSTGENOMIC TECHNIQUES FOR GLOBAL ANALYSIS

The combination of multiplex and automatable experimental protocols, such as DNA arrays for the acquisition of biological data and of bioinformatics techniques for data management, enables the creation of models that provide the researcher with the capability to change the observation scale from the fine details of a few genes to the complexity created by dynamic interacting networks. As a means to predict and control microbial responses, living organisms can be modeled as biological systems that result from the integration of several cross-talking networks that determine their phenotypes, the blueprint of which is defined by their genomic sequences. These dynamic networks include the subset of genes that are transcribed under a given set of conditions (transcriptome), the subset of transcripts that are expressed (proteome), the subset of reactions that occur in the cell (metabolome) and the resulting metabolite concentrations, the corresponding arrays of metabolic fluxes (fluxome), and a regulatory network (regulome) that captures external as well as internal stimuli and translates them into biological molecular commands. While technological progress still needs to be made to interpret in a global manner vast amounts of data and to recognize and understand new patterns of complexity, significant progress has already been achieved since the onset of the postgenomic era. Advances in this area have been made particularly in corynebacterial research, as exemplified by the generation of high-resolution two-dimensional electrophoresis proteome maps of the soluble proteins (44, 104), membrane proteins (105), and phosphoproteins (9) of *C. glutamicum*. The absence of a linear relationship between transcript abundance and enzyme activities (36) makes proteome analyses important complements to DNA array-generated transcription profiles to define global microbial responses to a variety of environmental conditions,

FIG. 1. Derivation of efficient bioconverters by postgenomic tools. The deciphering of the complete genomes of several strains of corynebacteria and the development of postgenomic tools, such as global analytical techniques, including global transposon mutagenesis, DNA microarrays, and proteomics, enable improved rational design in these microorganisms. The knowledge gathered by the identification of the functions of the individual genes can be completed by metabolic fluxes and networks, as well as transcription pattern analyses, under a variety of environmental conditions and subsequently leveraged by classical recombinant DNA techniques. The development of chromosome-engineering tools, such as megabase deletion and insertion or genome shuffling, makes possible genome streamlining and directed strain evolution procedures in these bacteria. In these methods, the iterative process of natural evolution can be mimicked at an accelerated pace to generate production strains tailored to the targeted industrial process by using selection conditions closely modeling the conditions of intended industrial use. This concept constitutes perhaps less a paradigm shift than an expansion of the traditional approach of random mutagenesis and positive selection by resistance to amino acid analogs to derive improved amino acid producers. However, the possibility to combine modern tools of engineering by rational design with evolutionary procedures breaks free from the constraints of traditional development strategies that involve only a few genes or a few parameters at a time and thus has the potential to enable a dramatic shift in production efficiencies of corynebacteria. To this end, systems biology and in silico cellular models, such as virtual cells, are expected to play an increasing role in both fundamental and applied science as the technology to integrate various dynamic physiological networks matures. The solid arrows indicate the forward direction of strain evolution along the axes of platform technology development and knowledge gathering. The broken arrows represent iterations in the evolution process that are necessary to fix multiple positive mutations. Fusion techniques are particularly important in this process, as they allow sexual evolution. The main arrows symbolize the combination of directed strain evolution and rational design to derive industrial strains with improved conversion capabilities.

such as heat shock (6) or ammonium limitation (108). On the other hand, initial attempts to increase metabolic fluxes via the overexpression of a limited number of key genes has yielded disappointing results, as metabolic imbalances thus created often result in unpredictable physiological responses and consequently in suboptimal metabolite productivities (64, 65). However, this hurdle can be circumvented by reestablishing the metabolic balance by the coordinated expression of most of the genes relevant to a production pathway while taking account of their respective global contributions to the cellular physiology (64, 65). Furthermore, the network topology of the various active reactions can be quantified by ^{13}C nuclear magnetic resonance to compare the performances of various isolates (32) or industrial fermentation conditions (62, 141). The integration of these different techniques has been successful, not only to rapidly delineate novel fundamental aspects of the

microbial metabolism of less researched species (32) or to engineer *C. glutamicum* strains toward increased production of, for example, lysine (67) or pantothenate (47), but also to reveal heretofore-undetected productivity constraints (47). The next frontier remains, however, the development of complete in silico models that would allow testing of hypotheses first in virtual cells and at a pace faster than can be achieved in vivo (53, 91).

A LOOK FORWARD

The objective to use whole microbial cells to produce fine chemicals, such as amino acids (43, 68) and therapeutic proteins or specialty enzymes (18a, 21, 41) that have a high value added, has already been achieved. The spectrum of products that can be synthesized by biotechnological processes seems

endless, provided the corresponding metabolic genes are available. However, in order to produce commodity biochemicals, and thus expand the industrial scope and societal impact of biotechnology, the economics of biotechnological processes need to be further improved to compete with the apparent cost achieved by conventional chemical synthesis. As a result, there is still a need to optimize the efficiency of microbial technology, even if these improvements offer only marginal cost-effectiveness, as the impacts of small incremental improvements are greatly amplified by large production scales. To strengthen the biotechnological attributes of *C. glutamicum*, whose potential to serve as a biocatalyst of commodity chemicals has recently been demonstrated (49, 52), the versatility of the corynebacterial transcription/translation system (92, 93) offers the possibility to adapt tools developed in *E. coli* and *B. subtilis*. However, the further improvement of corynebacterium-mediated bioconversions requires a greater understanding of corynebacterial physiology, such as catabolite repression or the fine details of the global regulation of gene expression and protein activity. Postgenomic tools and the recently acquired capability to manipulate coryneform bacteria at the genome level offer new avenues of research and development, not only in industrial microbiology, but also in fundamental research. For example, these developments will facilitate the understanding of the physiology of commensal and pathogenic corynebacterial species by defining the subset of genes that enable these bacteria to flourish in a different ecological niche than their closely related soil counterparts. Moreover, to develop strains that are cost-effective, genome streamlining to include only those pathways necessary for survival and the targeted industrial application is becoming increasingly attractive. Combined with the ability to control cellular division, metabolic streamlining could enable the engineering of industrial strains modeled on traditional factories where processes and flows can be controlled to maximize output. An added benefit derived from a simplified metabolism generating fewer by-products is the fact that the greater product purity that could be achieved would directly translate into savings at the downstream processing stage. However, genome size reduction alone to achieve metabolic streamlining may offer only an incomplete answer, since industrialization and the process robustness properties of the resulting chimeric organism could be severely negatively affected. We thus propose to explore a complementary strategy (Fig. 1) akin to directed-evolution techniques that have been successfully employed to optimize various enzymes (4). Conceivably, iterations of, for example, transposition-recombination-deletion-selection cycles could be used to derive cell catalysts not only with optimized general properties, but also with properties tailored to each particular industrial process. A key parameter in such directed strain evolution experiments is the ability to balance the overall fitness of the organism and genome rearrangements. Moreover, this method can be coupled with protoplast fusion and genome-shuffling methods that have been demonstrated to enable the rapid generation of strains with improved combined genetic properties (142). It nonetheless remains noteworthy that strains comprising a greatly reduced number of genes have the potential to serve as megabase gene delivery vesicles through protoplast fusion. Variations and combinations of these various techniques would allow us to mimic the natural evolution process (3, 27, 39, 101,

130), but at an accelerated pace, with fuzzily controlled cycles of deletions, insertions, duplications, and horizontal gene transfer in a context that replicates the intended conditions of use, with the ultimate goal to further increase our fundamental understanding of microbial physiology and to address various economic constraints that currently restrict the industrial scope of applied microbiology.

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REFERENCES

1. Anagnostopoulos, C. 1990. Genetic rearrangements in *Bacillus subtilis*, p. 361–367. In K. Drlica and M. Riley (ed.), *The bacterial chromosome*. American Society for Microbiology, Washington, D.C.
2. Ankri, S., O. Reyes, and G. Leblon. 1996. Electroporation of highly DNA-restrictive corynebacteria with synthetic DNA. *Plasmid* 35:62–66.
3. Arber, W. 2000. Genetic variation: molecular mechanisms and impact on microbial evolution. *FEMS Microbiol. Rev.* 24:1–7.
4. Arnold, F. H., and G. Georgiou. 2003. Directed enzyme evolution: screening and selection methods. Humana Press, Totowa, N.J.
5. Bardonnet, N., and C. Blanco. 1991. Improved vectors for transcriptional signal screening in corynebacteria. *FEMS Microbiol. Lett.* 68:97–102.
6. Barreiro, C., E. Gonzalez-Lavado, S. Brand, A. Tauch, and J. F. Martin. 2005. Heat shock proteome analysis of wild-type *Corynebacterium glutamicum* ATCC 13032 and a spontaneous mutant lacking GroEL1, a dispensable chaperone. *J. Bacteriol.* 187:884–889.
7. Barry, C. E., III, R. E. Lee, K. Mdluli, A. E. Sampson, B. G. Schroeder, R. A. Slayden, and Y. Yuan. 1998. Mycolic acids: structure, biosynthesis and physiological functions. *Prog. Lipid Res.* 37:143–179.
8. Behr, M. A., M. A. Wilson, W. P. Gill, H. Salamon, G. K. Schoolnik, S. Rane, and P. M. Small. 1999. Comparative genomics of BCG vaccines by whole-genome DNA microarray. *Science* 284:1520–1523.
9. Bendt, A. K., A. Burkovski, S. Schaffer, M. Bott, M. Farwick, and T. Hermann. 2003. Towards a phosphoproteome map of *Corynebacterium glutamicum*. *Proteomics* 3:1637–1646.
10. Billman-Jacobe, H., L. Wang, A. Kortt, D. Stewart, and A. Radford. 1995. Expression and secretion of heterologous proteases by *Corynebacterium glutamicum*. *Appl. Environ. Microbiol.* 61:1610–1613.
11. Bisno, A. L. 2004. Cutaneous infections: microbiologic and epidemiologic considerations. *Am. J. Med.* 76:172–179.
12. Bonamy, C., A. Guyonvarch, O. Reyes, F. David, and G. Leblon. 1990. Interspecies electro-transformation in *Corynebacteria*. *FEMS Microbiol. Lett.* 54:263–269.
13. Bonamy, C., J. Labarre, L. Cazaubon, C. Jacob, F. Le Bohec, O. Reyes, and G. Leblon. 2003. The mobile element IS1207 of *Brevibacterium lactofermentum* ATCC21086: isolation and use in the construction of Tn5531, a versatile transposon for insertional mutagenesis of *Corynebacterium glutamicum*. *J. Biotechnol.* 104:301–309.
14. Bonamy, C., J. Labarre, O. Reyes, and G. Leblon. 1994. Identification of IS1206, a *Corynebacterium glutamicum* IS3-related insertion sequence and phylogenetic analysis. *Mol. Microbiol.* 14:571–581.
15. Bonnassie, S., J. F. Burini, J. Oreglia, A. Trautwetter, J. C. Patte, and A. M. Sicard. 1990. Transfer of plasmid DNA to *Brevibacterium lactofermentum* by electroporation. *J. Gen. Microbiol.* 136:2107–2112.
16. Bonnassie, S., J. Oreglia, A. Trautwetter, and A. M. Sicard. 1990. Isolation and characterization of a restriction and modification deficient mutant of *Brevibacterium lactofermentum*. *FEMS Microbiol. Lett.* 60:143–146.
17. Cadenas, R. F., J. F. Martin, and J. A. Gil. 1991. Construction and characterization of promoter-probe vectors for *Corynebacteria* using the kanamycin-resistance reporter gene. *Gene* 98:117–121.
18. Cerdeño-Tarraga, A. M., A. Efratou, L. G. Dover, M. T. Holden, M. Pallen, S. D. Bentley, G. S. Besra, C. Churcher, K. D. James, A. De Zoysa, T. Chillingworth, A. Cronin, L. Dowd, T. Feltwell, N. Hamlin, S. Holroyd, K. Jagels, S. Moule, M. A. Quail, E. Rabinowitch, K. M. Rutherford, N. R. Thomson, L. Unwin, S. Whitehead, B. G. Barrell, and J. Parkhill. 2003. The complete genome sequence and analysis of *Corynebacterium diphtheriae* NCTC13129. *Nucleic Acids Res.* 31:6516–6523.
- 18a. Chance, R., R. Di Marchi, B. Frank, and J. E. Shields. June 1997. U.S. patent 5,700,662.
19. Chevalier, J., M. T. Pommier, A. Cremieux, and G. Michel. 1988. Influence of Tween 80 on the mycolic acid composition of three cutaneous corynebacteria. *J. Gen. Microbiol.* 134:2457–2461.
20. Correia, A., A. Pisabarro, J. M. Castro, and J. F. Martin. 1996. Cloning and

- characterization of an IS-like element present in the genome of *Brevibacterium lactofermentum* ATCC 13869. *Gene* **170**:91–94.
21. Date, M., K. Yokoyama, Y. Umezawa, H. Matsui, and Y. Kikuchi. 2004. High level expression of *Streptomyces mobaraensis* transglutaminase in *Corynebacterium glutamicum* using a chimeric pro-region from *Streptomyces cinnamomeus* transglutaminase. *J. Biotechnol.* **110**:219–226.
 22. de Boer, H. A., L. J. Comstock, and M. Vasser. 1983. The *tac* promoter: a functional hybrid derived from the *trp* and *lac* promoters. *Proc. Natl. Acad. Sci. USA* **80**:21–25.
 23. Deb, J. K., S. Malik, V. K. Ghosh, S. Mathai, and R. Sethi. 1990. Intergenic protoplast fusion between xylanase producing *Bacillus subtilis* LYT and *Corynebacterium acetoacidophilum* ATCC 21476. *FEMS Microbiol. Lett.* **59**:287–292.
 24. Deb, J. K., and N. Nath. 1999. Plasmids of corynebacteria. *FEMS Microbiol. Lett.* **175**:11–20.
 25. Dedonder, R. 1966. Levanucrase from *Bacillus subtilis*. *Methods Enzymol.* **8**:500–505.
 26. Delaunay, S., P. Gourdon, P. Lapujade, E. Mailly, E. Oriol, J. M. Engasser, N. D. Lindley, and J. L. Goergen. 1999. An improved temperature-triggered process for glutamate production with *Corynebacterium glutamicum*. *Enzyme Microb. Technol.* **25**:762–768.
 27. Dykhuizen, D. E. 1990. Experimental studies of natural selection in bacteria. *Annu. Rev. Ecol. Syst.* **21**:373–398.
 28. Eggeling, L., and O. Reyes. 2005. Experiments, p. 535–566. In L. Eggeling and M. Bott (ed.), *Handbook of Corynebacterium glutamicum*. CRC Press, Boca Raton, Fla.
 29. Eggeling, L., and H. Sahm. 1999. Amino acid production: principles of metabolic engineering, p. 153–176. In S. Y. Lee and E. T. Papoutsakis (ed.), *Metabolic Engineering*. Marcel Dekker, Inc., New York, N.Y.
 30. Ehrlich, S. D. 1989. Illegitimate recombination in bacteria, p. 799–832. In D. E. Berg and M. M. Howe (ed.), *Mobile DNA*. American Society for Microbiology, Washington, D.C.
 31. Eikmanns, B. J., E. Kleinertz, W. Liebl, and H. Sahm. 1991. A family of *Corynebacterium glutamicum*/*Escherichia coli* shuttle vectors for cloning, controlled gene expression, and promoter probing. *Gene* **102**:93–98.
 32. Fuhrer, T., E. Fischer, and U. Sauer. 2005. Experimental identification and quantification of glucose metabolism in seven bacterial species. *J. Bacteriol.* **187**:1581–1590.
 33. Funke, G., A. von Graevenitz, J. E. Clarridge III, and K. A. Bernard. 1997. Clinical microbiology of coryneform bacteria. *Clin. Microbiol. Rev.* **10**:125–159.
 34. Garbe, T. R., N. Suzuki, M. Inui, and H. Yukawa. 2004. Inhibitor-associated transposition events in *Corynebacterium glutamicum*. *Mol. Gen. Genom.* **271**:729–741.
 35. Gay, P., D. Le Coq, M. Steinmetz, T. Berkelman, and C. I. Kado. 1985. Positive selection procedure for entrapment of insertion sequence elements in gram-negative bacteria. *J. Bacteriol.* **164**:918–921.
 36. Glanemann, C., A. Loos, N. Gorret, L. B. Willis, X. M. O'Brien, P. A. Lessard, and A. J. Sinskey. 2003. Disparity between changes in mRNA abundance and enzyme activity in *Corynebacterium glutamicum*: implications for DNA microarray analysis. *Appl. Microbiol. Biotechnol.* **61**:61–68.
 37. Gokhale, D. V., U. S. Puntambekar, and D. N. Deobagkar. 1993. Protoplast fusion: a tool for intergeneric gene transfer in bacteria. *Biotechnol. Adv.* **11**:199–217.
 38. Goryshin, I. Y., and W. S. Reznikoff. 1998. Tn5 in vitro transposition. *J. Biol. Chem.* **273**:7367–7374.
 39. Hall, B. G. 1999. Toward an understanding of evolutionary potential. *FEMS Microbiol. Lett.* **178**:1–6.
 40. Hammes, W., K. H. Schleifer, and O. Kandler. 1973. Mode of action of glycine on the biosynthesis of peptidoglycan. *J. Bacteriol.* **116**:1029–1053.
 41. Hanquier, J., Y. Sorlet, D. Desplancq, L. Baroche, M. Ebtinger, J. F. Lefevre, F. Pattus, C. L. Hershberger, and A. A. Vertès. 2003. A single mutation in the activation site of bovine trypsinogen enhances its accumulation in the fermentation broth of the yeast *Pichia pastoris*. *Appl. Environ. Microbiol.* **69**:1108–1113.
 42. Haynes, J. A., and M. L. Britz. 1989. Electrotransformation of *Brevibacterium lactofermentum* and *Corynebacterium glutamicum*: growth in Tween 80 increases transformation frequencies. *FEMS Microbiol. Lett.* **61**:329–333.
 43. Hermann, T. 2003. Industrial production of amino acids by coryneform bacteria. *J. Biotechnol.* **104**:155–172.
 44. Hermann, T., W. Pfefferle, C. Baumann, E. Busker, S. Schaffer, M. Bott, H. Sahm, N. Dusch, J. Kalinowski, A. Pühler, A. K. Bendt, R. Kramer, and A. Burkowski. 2001. Proteome analysis of *Corynebacterium glutamicum*. *Electrophoresis* **22**:1712–1723.
 45. Horton, R. M. 1995. PCR-mediated recombination and mutagenesis. SOEing together tailor-made genes. *Mol. Biotechnol.* **3**:93–99.
 46. Hüser, A. T., A. Becker, I. Brune, M. Dondrup, J. Kalinowski, J. Plassmeier, A. Pühler, I. Wiegäbe, and A. Tauch. 2003. Development of a *Corynebacterium glutamicum* DNA microarray and validation by genome-wide expression profiling during growth with propionate as carbon source. *J. Biotechnol.* **106**:269–286.
 47. Hüser, A. T., C. Chassagnole, N. D. Lindley, M. Merkamm, A. Guyonvarch, V. Elisakova, M. Pátek, J. Kalinowski, I. Brune, A. Pühler, and A. Tauch. 2005. Rational design of a *Corynebacterium glutamicum* pantothenate production strain and its characterization by metabolic flux analysis and genome-wide transcriptional profiling. *Appl. Environ. Microbiol.* **71**:3255–3268.
 48. Ikeda, M., and S. Nakagawa. 2003. The *Corynebacterium glutamicum* genome: features and impacts on biotechnological processes. *Appl. Microbiol. Biotechnol.* **62**:99–109.
 49. Inui, M., H. Kawaguchi, S. Murakami, A. A. Vertès, and H. Yukawa. 2004. Metabolic engineering of *Corynebacterium glutamicum* for fuel ethanol production under oxygen deprivation conditions. *J. Mol. Microbiol. Biotechnol.* **8**:243–254.
 50. Inui, M., S. Murakami, S. Okino, H. Kawaguchi, A. A. Vertès, and H. Yukawa. 2004. Metabolic analysis of *Corynebacterium glutamicum* during lactate and succinate productions under oxygen deprivation conditions. *J. Mol. Microbiol. Biotechnol.* **7**:182–196.
 51. Inui, M., M. Terasawa, and H. Yukawa. 1999. L-Isoleucine, p. 1498–1503. In M. C. Flickinger and S. W. Drew (ed.), *Encyclopedia of bioprocess technology: fermentation, biocatalysis, and bioseparation*. John Wiley & Sons, New York, N.Y.
 52. Inui, M., Y. Tsuge, N. Suzuki, A. A. Vertès, and H. Yukawa. 2005. Isolation and characterization of a native composite transposon, Tn14751, carrying 17.4 kilobases of *Corynebacterium glutamicum* chromosomal DNA. *Appl. Environ. Microbiol.* **71**:407–416.
 53. Ishii, N., M. Robert, Y. Nakayama, A. Kanai, and M. Tomita. 2004. Toward large-scale modeling of the microbial cell for computer simulation. *J. Biotechnol.* **113**:281–294.
 54. Itaya, M., T. Nagata, T. Shiroishi, K. Fujita, and K. Tsuge. 2000. Efficient cloning and engineering of giant DNAs in a novel *Bacillus subtilis* genome vector. *J. Biochem.* **128**:869–875.
 55. Itaya, M., and T. Tanaka. 1997. Experimental surgery to create subgenomes of *Bacillus subtilis* 168. *Proc. Natl. Acad. Sci. USA* **94**:5378–5382.
 56. Jäger, W., A. Schäfer, J. Kalinowski, and A. Pühler. 1995. Isolation of insertion elements from gram-positive *Brevibacterium*, *Corynebacterium* and *Rhodococcus* strains using the *Bacillus subtilis* *sacB* gene as a positive selection marker. *FEMS Microbiol. Lett.* **126**:1–6.
 57. Jäger, W., A. Schäfer, A. Pühler, G. Labes, and W. Wohlleben. 1992. Expression of the *Bacillus subtilis* *sacB* gene leads to sucrose sensitivity in the gram-positive bacterium *Corynebacterium glutamicum* but not in *Streptomyces lividans*. *J. Bacteriol.* **174**:5462–5465.
 58. Jannière, L., A. Gruss, and S. D. Ehrlich. 1993. Plasmids, p. 625–644. In A. L. Sonenshein, J. A. Hoch, and R. Losick (ed.), *Bacillus subtilis* and other gram-positive bacteria. American Society for Microbiology, Washington, D.C.
 59. Kalinowski, J. 2004. The genomes of amino-acid producing corynebacteria, p. 37–56. In L. Eggeling and M. Bott (ed.), *Handbook of Corynebacterium glutamicum*. CRC Press, Boca Raton, Fla.
 60. Kalinowski, J., B. Bathe, D. Bartels, N. Bischoff, M. Bott, A. Burkowski, N. Dusch, L. Eggeling, B. J. Eikmanns, L. Gaigalat, A. Goesmann, M. Hartmann, K. Huthmacher, R. Krämer, B. Linke, A. C. McHardy, F. Meyer, B. Mockel, W. Pfefferle, A. Pühler, D. A. Rey, C. Ruckert, O. Rupp, H. Sahm, V. F. Wendisch, I. Wiegäbe, and A. Tauch. 2003. The complete *Corynebacterium glutamicum* ATCC 13032 genome sequence and its impact on the production of L-aspartate-derived amino acids and vitamins. *J. Biotechnol.* **104**:5–25.
 61. Kato-Maeda, M., J. T. Rhee, T. R. Gingeras, H. Salamon, J. Drenkow, N. Smittipat, and P. M. Small. 2001. Comparing genomes within the species *Mycobacterium tuberculosis*. *Genome Res.* **11**:547–554.
 62. Kiefer, P., E. Heinzle, O. Zelder, and C. Wittmann. 2004. Comparative metabolic flux analysis of lysine-producing *Corynebacterium glutamicum* cultured on glucose or fructose. *Appl. Environ. Microbiol.* **70**:229–239.
 63. Kirchner, O., and A. Tauch. 2003. Tools for genetic engineering in the amino acid-producing bacterium *Corynebacterium glutamicum*. *J. Biotechnol.* **104**:287–299.
 64. Koffas, M., and G. Stephanopoulos. 2005. Strain improvement by metabolic engineering: lysine production as a case study for systems biology. *Curr. Opin. Biotechnol.* **16**:361–366.
 65. Koffas, M. A., G. Y. Jung, and G. Stephanopoulos. 2003. Engineering metabolism and product formation in *Corynebacterium glutamicum* by co-ordinated gene overexpression. *Metab. Eng.* **5**:32–41.
 66. Krämer, R. 1994. Secretion of amino acids by bacteria: physiology and mechanism. *FEMS Microbiol. Rev.* **13**:75–94.
 67. Krömer, J. O., O. Sorgenfrei, K. Klopprogge, E. Heinzle, and C. Wittmann. 2004. In-depth profiling of lysine-producing *Corynebacterium glutamicum* by combined analysis of the transcriptome, metabolome, and fluxome. *J. Bacteriol.* **186**:1769–1784.
 68. Kumagai, H. 2000. Microbial production of amino acids in Japan. *Adv. Biochem. Eng. Biotechnol.* **69**:71–85.
 69. Kuru, Y., M. Kainuma, M. Inui, Y. Satoh, and H. Yukawa. 1990. Electroporation-transformation system for coryneform bacteria by auxotrophic complementation. *Agric. Biol. Chem.* **54**:443–447.
 70. Kuru, Y., Y. Satoh, M. Inui, K. Kohama, M. Kobayashi, M. Terasawa,

- and H. Yukawa. 1991. Identification of plasmid partition function in coryneform bacteria. *Appl. Environ. Microbiol.* **57**:759–764.
71. Lepesant, J. A., J. Lepesant-Kejzarova, M. Pascal, F. Kunst, A. Billault, and R. Dedonder. 1974. Identification of the structural gene of levansucrase in *Bacillus subtilis* Marburg. *Mol. Gen. Genet.* **128**:213–221.
 72. Liebl, W. 2004. *Corynebacterium* taxonomy, p. 9–34. In L. Eggeling and M. Bott (ed.), *Handbook of Corynebacterium glutamicum*. CRC Press, Boca Raton, Fla.
 73. Liebl, W., A. Bayerl, B. Schein, U. Stillner, and K. H. Schleifer. 1989. High efficiency electroporation of intact *Corynebacterium glutamicum* cells. *FEMS Microbiol. Lett.* **53**:299–303.
 74. Liebl, W., M. Ehrmann, W. Ludwig, and K. H. Schleifer. 1991. Transfer of *Brevibacterium divaricatum* DSM 20297^T, “*Brevibacterium flavum*” DSM 20411, “*Brevibacterium lactofermentum*” DSM 20412 and DSM 1412, and *Corynebacterium lilium* DSM 20137^T to *Corynebacterium glutamicum* and their distinction by rRNA gene restriction patterns. *Int. J. Syst. Bacteriol.* **41**:255–260.
 75. Mahillon, J., and M. Chandler. 1998. Insertion sequences. *Microbiol. Mol. Biol. Rev.* **62**:725–774.
 76. Martin, J. F., and J. A. Gil. 1999. Corynebacteria, p. 379–391. In A. L. Demain, J. E. Davies, R. M. Atlas, G. Cohen, C. L. Hershberger, W. S. Hu, D. H. Sherman, R. C. Wilson, and J. H. D. Wu (ed.), *Manual of industrial microbiology and biotechnology*, 2 ed. American Society for Microbiology, Washington, D.C.
 77. Miller, M. L. 1994. A field study evaluating the effectiveness of different hand soaps and sanitizers. *Dairy Food Environ. Sanitation* **14**:155–160.
 78. Mira, A., H. Ochman, and N. A. Moran. 2001. Deletional bias and the evolution of bacterial genomes. *Trends Genet.* **17**:589–596.
 79. Mirdamadi-Tehrani, J., J. I. Mitchell, S. T. Williams, and D. A. Ritchie. 1992. Interspecific protoplast fusion and genetic recombination between *Streptomyces griseus* and *Streptomyces griseolus*. *FEMS Microbiol. Lett.* **91**:187–192.
 80. Moreau, S., C. Blanco, and A. Trautwetter. 1999. Site-specific integration of corynephage ϕ 16: construction of an integration vector. *Microbiology* **145**:539–548.
 81. Moreau, S., V. Leret, C. Le Marrec, H. Varangot, M. Ayache, S. Bonnassie, C. Blanco, and A. Trautwetter. 1995. Prophage distribution in coryneform bacteria. *Res. Microbiol.* **146**:493–505.
 82. Morinaga, Y., M. Tsuchiya, K. Miwa, and K. Sano. 1987. Expression of *Escherichia coli* promoters in *Brevibacterium lactofermentum* using the shuttle vector pEB003. *J. Biotechnol.* **5**:305–312.
 83. Muffler, A., S. Bettermann, M. Haushalter, A. Horlein, U. Neveling, M. Schramm, and O. Sorgenfrei. 2002. Genome-wide transcription profiling of *Corynebacterium glutamicum* after heat shock and during growth on acetate and glucose. *J. Biotechnol.* **98**:255–268.
 84. Mukherjee, K. J., J. K. Deb, and K. B. Ramachandran. 1996. Studies on maintenance of vector pBK2 in *Corynebacterium acetoacidophilum* by co-culture experiments in a continuous bioreactor. *Enzyme Microb. Technol.* **18**:555–560.
 85. Nakamura, Y., Y. Nishio, K. Ikeo, and T. Gojobori. 2003. The genome stability in *Corynebacterium* species due to lack of the recombinational repair system. *Gene* **317**:149–155.
 86. Nakata, K., M. Inui, P. B. Kós, A. A. Vertès, and H. Yukawa. 2004. Vectors for genetic engineering of corynebacteria, p. 175–191. In B. C. Saha (ed.), *Fermentation biotechnology*. American Chemical Society, Washington, D.C.
 87. Nishio, Y., Y. Nakamura, Y. Kawarabayasi, Y. Usuda, E. Kimura, S. Sugimoto, K. Matsui, A. Yamagishi, H. Kikuchi, K. Ikeo, and T. Gojobori. 2003. Comparative complete genome sequence analysis of the amino acid replacements responsible for the thermostability of *Corynebacterium efficiens*. *Genome Res.* **13**:1572–1579.
 88. Ohnishi, J., S. Mitsuhashi, M. Hayashi, S. Ando, H. Yokoi, K. Ochiai, and M. Ikeda. 2002. A novel methodology employing *Corynebacterium glutamicum* genome information to generate a new L-lysine-producing mutant. *Appl. Microbiol. Biotechnol.* **58**:217–223.
 89. Oram, D. M., A. Avdalovic, and R. K. Holmes. 2002. Construction and characterization of transposon insertion mutations in *Corynebacterium diphtheriae* that affect expression of the diphtheria toxin repressor (DtxR). *J. Bacteriol.* **184**:5723–5732.
 90. Pandey, A., and M. Mann. 2000. Proteomics to study genes and genomes. *Nature* **405**:837–846.
 91. Papin, J. A., N. D. Price, and B. O. Palsson. 2004. *In silico* cells: studying genotype-phenotype relationships using constraint-based models, p. 209–236. In B. N. Kholodenko and H. V. Westerhoff (ed.), *Metabolic engineering in the post genomic era*. Horizon Bioscience, Wymondham, United Kingdom.
 92. Pátek, M., G. Muth, and W. Wohlleben. 2003. Function of *Corynebacterium glutamicum* promoters in *Escherichia coli*, *Streptomyces lividans*, and *Bacillus subtilis*. *J. Biotechnol.* **104**:325–334.
 93. Pátek, M., J. Nesvera, A. Guyonvarch, O. Reyes, and G. Leblon. 2003. Promoters of *Corynebacterium glutamicum*. *J. Biotechnol.* **104**:311–323.
 94. Peberdy, J. F. 1980. Protoplast fusion—a tool for genetic manipulation and breeding in industrial microorganisms. *Enzyme Microb. Technol.* **2**:23–29.
 95. Quast, K., B. Bathe, A. Pühler, and J. Kalinowski. 1999. The *Corynebacterium glutamicum* insertion sequence ISCG2 prefers conserved target sequences located adjacent to genes involved in aspartate and glutamate metabolism. *Mol. Gen. Genet.* **262**:568–578.
 96. Rappuoli, R., M. Perugini, and G. Ratti. 1987. DNA element of *Corynebacterium diphtheriae* with properties of an insertion sequence and usefulness for epidemiological studies. *J. Bacteriol.* **169**:308–312.
 97. Reyes, O., A. Guyonvarch, C. Bonamy, V. Salti, F. David, and G. Leblon. 1991. ‘Integron’-bearing vectors: a method suitable for stable chromosomal integration in highly restrictive corynebacteria. *Gene* **107**:61–68.
 98. Ruimy, R., P. Riegel, P. Boiron, H. Monteil, and R. Christen. 1995. Phylogeny of the genus *Corynebacterium* deduced from analyses of small-subunit ribosomal DNA sequences. *Int. J. Syst. Bacteriol.* **45**:740–746.
 99. Sahm, H., L. Eggeling, and A. A. de Graaf. 2000. Pathway analysis and metabolic engineering in *Corynebacterium glutamicum*. *Biol. Chem.* **381**:899–910.
 100. Salim, K., V. Haedens, J. Content, G. Leblon, and K. Huygen. 1997. Heterologous expression of the *Mycobacterium tuberculosis* gene encoding antigen 85A in *Corynebacterium glutamicum*. *Appl. Environ. Microbiol.* **63**:4392–4400.
 101. Sauer, U. 2001. Evolutionary engineering of industrially important microbial phenotypes. *Adv. Biochem. Eng. Biotechnol.* **73**:129–169.
 102. Schäfer, A., A. Tauch, N. Droste, A. Pühler, and J. Kalinowski. 1997. The *Corynebacterium glutamicum* cglIM gene encoding a 5-cytosine methyltransferase enzyme confers a specific DNA methylation pattern in an MCRBC-deficient *Escherichia coli* strain. *Gene* **203**:95–101.
 103. Schäfer, A., A. Tauch, W. Jäger, J. Kalinowski, G. Thierbach, and A. Pühler. 1994. Small mobilizable multi-purpose cloning vectors derived from the *Escherichia coli* plasmids pK18 and pK19: selection of defined deletions in the chromosome of *Corynebacterium glutamicum*. *Gene* **145**:69–73.
 104. Schaffer, S., B. Weil, V. D. Nguyen, G. Dongmann, K. Günther, M. Nickolaus, T. Hermann, and M. Bott. 2001. A high-resolution reference map for cytoplasmic and membrane-associated proteins of *Corynebacterium glutamicum*. *Electrophoresis* **22**:4404–4422.
 105. Schluesener, D., F. Fischer, J. Kruij, M. Rögnér, and A. Poetsch. 2005. Mapping the membrane proteome of *Corynebacterium glutamicum*. *Proteomics* **5**:1317–1330.
 106. Schumann, W., and C. S. Ferreira. 2004. Production of recombinant proteins in *Escherichia coli*. *Genet. Mol. Biol.* **27**:442–453.
 107. Schwarzer, A., and A. Pühler. 1991. Manipulation of *Corynebacterium glutamicum* by gene disruption and replacement. *Biotechnology* **9**:84–87.
 108. Silberbach, M., M. Schäfer, A. T. Hüser, J. Kalinowski, A. Pühler, R. Krämer, and A. Burkovski. 2005. Adaptation of *Corynebacterium glutamicum* to ammonium limitation: a global analysis using transcriptome and proteome techniques. *Appl. Environ. Microbiol.* **71**:2391–2402.
 109. Simic, P., H. Sahm, and L. Eggeling. 2001. L-Threonine export: use of peptides to identify a new translocator from *Corynebacterium glutamicum*. *J. Bacteriol.* **183**:5317–5324.
 110. Srivastava, P., and J. K. Deb. 2005. Gene expression systems in corynebacteria. *Protein Expr. Purif.* **40**:221–229.
 111. Stackebrandt, E., F. A. Rainey, and L. Naomi. 1997. Proposal for a new hierarchic classification system, Actinobacteria classis nov. *Int. J. Syst. Bacteriol.* **47**:479–791.
 112. Stratton, H. M., P. R. Brooks, E. L. Carr, and R. J. Seviour. 2003. Effects of culture conditions on the mycolic acid composition of isolates of *Rhodococcus* spp. from activated sludgefoams. *Syst. Appl. Microbiol.* **26**:165–171.
 113. Sulakvelidze, A., M. Kekelidze, T. Gomelauri, Y. Deng, N. Khetsuriani, K. Kobaidze, A. De Zoysa, A. Efstratiou, J. G. Morris, Jr., and P. Imnadze. 1999. Diphtheria in the Republic of Georgia: use of molecular typing techniques for characterization of *Corynebacterium diphtheriae* strains. *J. Clin. Microbiol.* **37**:3265–3270.
 114. Suzuki, N., H. Nonaka, Y. Tsuge, S. Okayama, M. Inui, and H. Yukawa. 21 April 2005. Multiple large segment deletion method for *Corynebacterium glutamicum*. *Appl. Microbiol. Biotechnol.* **10.1007/s00253-005-1976-4**.
 115. Suzuki, N., S. Okayama, H. Nonaka, Y. Tsuge, M. Inui, and H. Yukawa. 2005. Large-scale engineering of the *Corynebacterium glutamicum* genome. *Appl. Environ. Microbiol.* **71**:3369–3372.
 116. Suzuki, N., Y. Tsuge, M. Inui, and H. Yukawa. 2005. *cre/loxP*-mediated deletion system for large genome rearrangements in *Corynebacterium glutamicum*. *Appl. Microbiol. Biotechnol.* **67**:225–233.
 117. Takayama, K., B. Hayes, M. M. Vestling, and R. J. Massey. 2003. Transposon-5 mutagenesis transforms *Corynebacterium matruchotii* to synthesize novel hybrid fatty acids that functionally replace corynomycolic acid. *Biochem. J.* **373**:465–474.
 118. Tauch, A., N. Bischoff, I. Brune, and J. Kalinowski. 2003. Insights into the genetic organization of the *Corynebacterium diphtheriae* erythromycin resistance plasmid pNG2 deduced from its complete nucleotide sequence. *Plasmid* **49**:63–74.
 119. Tauch, A., N. Bischoff, A. Pühler, and J. Kalinowski. 2004. Comparative genomics identified two conserved DNA modules in a corynebacterial plas-

- mid family present in clinical isolates of the opportunistic human pathogen *Corynebacterium jeikeium*. Plasmid **52**:102–118.
120. Tauch, A., S. Götker, A. Pühler, J. Kalinowski, and G. Thierbach. 2002. The 27.8-kb R-plasmid pTET3 from *Corynebacterium glutamicum* encodes the aminoglycoside adenylyltransferase gene cassette *aadA9* and the regulated tetracycline efflux system Tet 33 flanked by active copies of the widespread insertion sequence IS6100. Plasmid **48**:117–129.
 121. Tauch, A., S. Götker, A. Pühler, J. Kalinowski, and G. Thierbach. 2002. The alanine racemase gene *alr* is an alternative to antibiotic resistance genes in cloning systems for industrial *Corynebacterium glutamicum* strains. J. Biotechnol. **99**:79–91.
 122. Tauch, A., O. Kaiser, T. Hain, A. Goesmann, B. Weisshaar, A. Albersmeier, T. Bekel, N. Bischoff, I. Brune, T. Chakraborty, J. Kalinowski, F. Meyer, O. Rupp, S. Schneiker, P. Viehöver, and A. Pühler. 2005. Complete genome sequence and analysis of the multiresistant nosocomial pathogen *Corynebacterium jeikeium* K411, a lipid-requiring bacterium of the human skin flora. J. Bacteriol. **187**:4671–4682.
 123. Tauch, A., F. Kassing, J. Kalinowski, and A. Pühler. 1995. The *Corynebacterium xerosis* composite transposon Tn5432 consists of two identical insertion sequences, designated IS1249, flanking the erythromycin resistance gene, *ermCX*. Plasmid **34**:119–131.
 124. Tauch, A., F. Kassing, J. Kalinowski, and A. Pühler. 1995. The erythromycin resistance gene of the *Corynebacterium xerosis* R-plasmid pTP10 also carrying chloramphenicol, kanamycin, and tetracycline resistances is capable of transposition in *Corynebacterium glutamicum*. Plasmid **33**:168–179.
 125. Tauch, A., O. Kirchner, B. Löffler, S. Götker, A. Pühler, and J. Kalinowski. 2002. Efficient electrotransformation of *Corynebacterium diphtheriae* with a mini-replicon derived from the *Corynebacterium glutamicum* plasmid pGA1. Curr. Microbiol. **45**:362–367.
 126. Tauch, A., S. Krieft, J. Kalinowski, and A. Pühler. 2000. The 51,409-bp R-plasmid pTP10 from the multiresistant clinical isolate *Corynebacterium striatum* M82B is composed of DNA segments initially identified in soil bacteria and in plant, animal, and human pathogens. Mol. Gen. Genet. **263**:1–11.
 127. Tauch, A., A. Pühler, J. Kalinowski, and G. Thierbach. 2003. Plasmids in *Corynebacterium glutamicum* and their molecular classification by comparative genomics. J. Biotechnol. **104**:27–40.
 128. Tauch, A., Z. Zheng, A. Pühler, and J. Kalinowski. 1998. *Corynebacterium striatum* chloramphenicol resistance transposon Tn5564: genetic organization and transposition in *Corynebacterium glutamicum*. Plasmid **40**:126–139.
 129. Thatcher, J. W., J. M. Shaw, and W. J. Dickinson. 1998. Marginal fitness contributions of nonessential genes in yeast. Proc. Natl. Acad. Sci. USA **95**:253–257.
 130. Trevors, J. T. 1997. Evolution of bacterial genomes. Antonie Leeuwenhoek **71**:265–270.
 131. Tsuge, Y., K. Ninomiya, N. Suzuki, M. Inui, and H. Yukawa. 2005. A new insertion sequence, IS14999, from *Corynebacterium glutamicum*. Microbiology **151**:501–508.
 132. Valic, B., M. Golzio, M. Pavlin, A. Schatz, C. Faurie, B. Gabriel, J. Teissié, M. P. Rols, and D. Miklavcic. 2003. Effect of electric field induced transmembrane potential on spheroidal cells: theory and experiment. Eur. Biophys. J. **32**:519–528.
 133. van der Rest, M. E., C. Lange, and D. Molenaar. 1999. A heat shock following electroporation induces highly efficient transformation of *Corynebacterium glutamicum* with xenogeneic plasmid DNA. Appl. Microbiol. Biotechnol. **52**:541–545.
 134. Venkova, T., M. Pátek, and J. Nesvera. 2001. Identification of a novel gene involved in stable maintenance of plasmid pGA1 from *Corynebacterium glutamicum*. Plasmid **46**:153–162.
 135. Verma, V., G. N. Qazi, and R. Parshad. 1992. Intergeneric protoplast fusion between *Gluconobacter oxydans* and *Corynebacterium* species. J. Biotechnol. **26**:327–330.
 136. Vertès, A. A., Y. Asai, M. Inui, M. Kobayashi, Y. Kurusu, and H. Yukawa. 1994. Transposon mutagenesis of coryneform bacteria. Mol. Gen. Genet. **245**:397–405.
 137. Vertès, A. A., K. Hatakeyama, M. Inui, M. Kobayashi, Y. Kurusu, and H. Yukawa. 1993. Replacement recombination in coryneform bacteria: high efficiency integration requirement for non-methylated plasmid DNA. Biosci. Biotechnol. Biochem. **57**:2036–2038.
 138. Vertès, A. A., M. Inui, M. Kobayashi, Y. Kurusu, and H. Yukawa. 1994. Isolation and characterization of IS31831, a transposable element from *Corynebacterium glutamicum*. Mol. Microbiol. **11**:739–746.
 139. Vertès, A. A., M. Inui, M. Kobayashi, Y. Kurusu, and H. Yukawa. 1993. Presence of *mrr*- and *mcr*-like restriction systems in coryneform bacteria. Res. Microbiol. **144**:181–185.
 140. Wendisch, V. F. 2003. Genome-wide expression analysis in *Corynebacterium glutamicum* using DNA microarrays. J. Biotechnol. **104**:273–285.
 141. Wendisch, V. F., A. A. de Graaf, H. Sahm, and B. J. Eikmanns. 2000. Quantitative determination of metabolic fluxes during cointilization of two carbon sources: comparative analyses with *Corynebacterium glutamicum* during growth on acetate and/or glucose. J. Bacteriol. **182**:3088–3096.
 142. Zhang, Y. X., K. Perry, V. A. Vinci, K. Powell, W. P. Stemmer, and S. B. del Cardayré. 2002. Genome shuffling leads to rapid phenotypic improvement in bacteria. Nature **415**:644–646.
 143. Zimmermann, U., G. Pilwat, F. Beckers, and F. Rieman. 1976. Effects of external electric fields on cell membranes. Bioelectrochem. Bioenerg. **3**:58–83.
 144. Zupancic, T. J., J. D. Kittle, B. D. Baker, C. J. Miller, D. T. Palmer, Y. Asai, M. Inui, A. Vertès, M. Kobayashi, Y. Kurusu, and H. Yukawa. 1995. Isolation of promoters from *Brevibacterium flavum* strain MJ233C and comparison of their gene expression levels in *B. flavum* and *Escherichia coli*. FEMS Microbiol. Lett. **131**:121–126.