Involvement of Enterobactin Synthesis Pathway in Production of Microcin H47

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Microcin H47 (MccH47) is a gene-encoded peptide antibiotic produced by an *Escherichia coli* clinical isolate which is active on strains of gram-negative bacteria. Its uptake by *E. coli* K-12-susceptible cells depends on the presence of any of the outer membrane proteins Cir, Fiu, and FepA, the three catechol receptors of this organism. The nucleotide sequence of a portion of the MccH47 genetic system that had not yet been studied was elucidated. Five open reading frames were identified, three of which corresponded to genes encoding functions related to catechol-type siderophores: *mchA* and *mchS1* are *iroB* and *iroD* homologues, respectively, and *mchS4* was found to promote the production of the catecholate siderophore enterobactin. The possible relationship between enterobactin synthesis and MccH47 production was studied. Enterobactin-deficient strains failed to produce MccH47 when transformed with the antibiotic genetic determinants and upon introduction of the *ent* genetic cluster, the production of both the siderophore and MccH47 was restored. Further studies demonstrated that at least the enterobactin nonribosomal peptide synthase EntF is necessary for MccH47 synthesis. The relationship found between MccH47 and catecholate siderophore production is discussed, and a model outlining MccH47 synthesis is proposed.

Microcins are a group of antibiotic peptides produced by strains of gram-negative bacteria. They are synthesized as gene-encoded peptides that thereafter may undergo posttranslational modifications to be converted into mature molecules (3, 12).

Microcin H47 (MccH47) is produced by *Escherichia coli* strain H47, a clinical isolate from human feces, and is active on bacteria phylogenetically related to the producing strain. The MccH47 genetic system was located in the chromosome, extending over a ca. 10-kb DNA segment (14) (Fig. 1a). It comprises four genes involved in microcin synthesis (one of them being the microcin structural gene), two further genes devoted to microcin secretion, and an immunity gene whose product protects the cell against its own antibiotic production. Within the genetic system, there is a DNA segment of ca. 3 kb where insertion mutations did not significantly affect the antibiosis phenotypes of production or immunity. This DNA segment was thus provisionally called the silent region (2, 8, 19, 20).

MccH47 is ribosomally synthesized as a 75-residue peptide precursor, the product of the synthesis gene *mchB*. The MchB peptide possesses deleterious activity of the same specificity as mature MccH47, but it differs from the latter by its inability to be taken in by cells. This deleterious activity is detected as a specific toxic effect exerted upon cells synthesizing MchB: the introduction of the single *mchB* gene into wild-type *E. coli* K12 cells renders them nonviable, with this situation reverting when cells also contain the MccH47 immunity gene *mchI*. To produce mature antibiotic activity, the presence of all four microcin synthesis genes, *mchA*, *mchB*, *mchC*, and *mchD*, is required. Therefore, it has been proposed that the products of

mchA, *-C*, and *-D* would impose posttranslational modifications upon the peptide precursor, thus rendering the molecule suitable for uptake (20). Finally, the antibiotic is secreted through an ABC export apparatus composed of the products of the *mchE* and *mchF* genes from the MccH47 system and by the product of the unlinked *tolC* gene. During secretion, the molecule would be processed in its 15 N-terminal residues (2, 20).

The mechanism of action of MccH47 on susceptible *E. coli* K-12 cells has been analyzed. Microcin uptake is mediated by any of the outer membrane receptors FepA, Fiu, and Cir, plus the TonB pathway; i.e., MccH47 makes use of the initial steps for catecholate siderophore uptake (7, 24). This finding was the first clue indicating that MccH47 and catecholate siderophores could be somehow related, a subject that will be developed in this work. Once inside susceptible cells, MccH47 would interact with the proton channel of ATP synthase, which was shown to be absolutely necessary for antibiotic action (20, 21, 24).

E. coli synthesizes and secretes siderophores, which are compounds that chelate ferric iron from the medium and, as Fe(III)-siderophore complexes, are then imported back into cells to provide this metal for metabolic processes. Enterobactin (also called enterochelin), the main siderophore of the catechol type produced by this organism, makes use of FepA as its receptor, while its precursor dihydroxybenzoic acid (DHB) and its breakdown product dihydroxybenzoylserine (DBS) can be taken up by FepA, Cir, or Fiu (10). The determinants for enterobactin synthesis, secretion, uptake, and hydrolysis are clustered in the *ent* system, which is located in the chromosome in *E. coli* and other enterobacteria (5, 7). An additional locus, *iroA*, has recently been found to be related to the production and uptake of a new type of catecholate siderophores named salmochelins. This locus was found in *Salmonella enterica* as well as in some pathogenic *E. coli* and *Klebsiella pneumoniae* strains. It comprises five genes, *iroBCDEN*, although in some

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FIG. 1. MccH47 genetic system. (a) DNA segment containing the entire MccH47 genetic system cloned into plasmid pEX100. Previously described genes with their directions of transcription are depicted below the segment. The extension of the MccH47 system and the silent region is indicated (8, 19). (b) Enlargement of the DNA segment analyzed in this work. A double bar shows the newly sequenced DNA. ORFs are shown below the segment. (c) DNA segments cloned into pUY51 and pMVDS4. C, ClaI; E, EcoRI; G, BglIII; H, HindIII; P, PstI.

organisms only a subset of them is present (4, 6, 11, 13, 18, 25). The *iroB* and *iroD* genes have been found to be related to the production of salmochelins. Sequence similarities indicate that IroB would be a glycosyltransferase and that IroD would be a ferric enterobactin esterase. Consistently, the basic structure of salmochelins was described as the enterobactin breakdown product DBS modified by a sugar addition. Salmochelins would thus be more hydrophilic than enterobactin, a feature that could facilitate iron uptake by pathogenic bacteria in their host (11).

The results presented in this work indicate that a strict correlation exists between the production of MccH47 and that of the siderophore enterobactin. It was found that strains carrying the MccH47 system overproduce enterobactin and that the enterobactin synthesis (Ent) pathway is required for antibiotic synthesis. The contribution of the Ent pathway would take place during microcin maturation, a stage where the molecule is rendered suitable for its uptake into susceptible cells. We consider the possibility that the Ent pathway could provide a catechol or a catechol-derived moiety for the construction of the antibiotic molecule and that this moiety would be responsible for MccH47 uptake through the catechol receptors Cir, Fiu, and FepA. In addition, the finding that the MccH47 genetic system contains *iroB* and *iroD* homologues suggests that the catechol-like molecule provided for MccH47 synthesis could be related to salmochelins.

MATERIALS AND METHODS

Media and culture conditions. Luria-Bertani (LB) rich medium and a modified M63 minimal medium (with no addition of iron) supplemented with glucose were used (15). To detect siderophore production, chrome azurol S (CAS) agar plates were employed (23). When required, L-amino acids were added at a final concentration of 40 µg/ml. Iron-rich media were prepared by the addition of 10 µg of $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ per ml; iron limitation was imposed by adding 47 µg of 2,2-dipyridyl per ml, and, when stated, plates were supplemented with 10 µg of 2,3-dihydroxybenzoic acid (DHB) per ml. Antibiotics were added to media at the following final concentrations in micrograms per milliliter: ampicillin, 50; chloramphenicol, 60; and kanamycin, 30.

Bacterial strains. *E. coli* K-12 strains from our laboratory collection (MC4100 [*araD* Δ *lacU169* *relA* *rpsL* *thiA*] and RYC1000 [*araD* *gyrA* Δ *lacU169* *recA* *rbs* *relA* *rpsL* *thiA*]) were used. The non-enterobactin-producing (Ent[−]) strain UT400 [*araC* *entA403* *lacY* *leuB* *mtl* Δ (*ompT-fepC*)267 *proC* *rfbD* *rpsL* *secA* *thi* *trpE* *tsx* *xylA*] is CGSC 7231 (Genetic Stock Center). Two strains were constructed by P1 transduction (15): FGB102, a *fepA*::Tn10 transductant of MC4100 infected with P1 grown on H1876 (10), and MC4100 Δ (*ompT-fepC*)267, whose deletion proceeds from UT400. The *ent* genetic structure of MC4100 Δ (*ompT-fepC*)267 was confirmed by the ability of plasmid pUY47 (see below) to complement the deletion mutation for enterobactin production.

Plasmids used and plasmid construction. Previously described plasmids were used: pEX100, containing the MccH47 genetic system (8); pUY69, bearing the MccH47 immunity gene *mchI* (19), and pMVD10, carrying the MccH47 structural gene *mchB* (20). Plasmid pMVDS4, carrying only *mchS4*, was obtained by subcloning a 659-bp ClaI DNA fragment from pEX100 into pACYC184. Plasmid pUY51, bearing only *mchS1*, was constructed by subcloning a 1,905-bp PstI-BglII DNA fragment from pEX100 into the pUC13 vector. The entire *ent* system from the chromosome of the Ent⁺ strain MC4100 was cloned in vivo by means of lytic propagation of phage Mud5005 in this strain, as described previously (9, 14). The lysate was employed to infect the Ent[−] strain UT400, which was previously

lysogenized with phage *Mu*cts Ap^r (8), and transductants were selected on LB plates supplemented with kanamycin and 2,2-dipyridyl. The plasmid carried by one transductant, pENT1, harbored the entire *ent* system included in an insert of about 27 kb. Several DNA segments of the *ent* system were subcloned from pENT1: pUY47 is a pBR322 derivative with a 10,817-bp HindIII insert containing *entD*, *fepA*, *fes*, and *entF*; pUY46 contains *entD*, *fepA*, and *fes* in a 6,265-bp BamHI insert cloned into the same vector; and pUY44 bears only the *fes* gene, included in a 2,991-bp EcoRI fragment cloned into pUC13.

MccH47 production assay and MccH47 extracts. MccH47 production was assayed by patch test on minimal medium plates as described previously (20). The detection of intracellular MccH47 activity was performed by cell lysis (8). To assay iron-chelating activity of MccH47 extracts, these were also prepared by cell lysis as before, but with water instead of buffer in order to avoid chelating interferences; their antibiotic activity was confirmed by a spot inhibition test on a lawn of an MccH47-sensitive strain. The indicator strains used were RYC1000(pUC13) (MccH47 sensitive) and RYC1000(pUY69) (MccH47 immune). MccH47 activity generates antibiosis halos on the former and has no effect on the latter.

Siderophore production assay. Strains to be assayed were stabbed with a sterile toothpick on CAS plates, in which iron binds to chrome azurol S, conferring a dark green color to the medium. In the presence of siderophore, regardless of its chemical nature, iron binds the chelator instead of CAS and the color turns yellow. In the assay, siderophore production was detected by the appearance of yellow halos around the stabs after 24 h of incubation.

DNA manipulation and sequencing. Routine DNA manipulations and plasmid vectors were employed (22). DNA sequencing was performed at the DNA Sequencing Core Laboratory Service of the University of Florida, Gainesville.

Nucleotide sequence accession number. The sequence of the DNA containing *mchA* through *mchS4* has been deposited in the EMBL database under accession no. AJ009631.

RESULTS

Nucleotide sequence determination and analysis. The nucleotide sequence of a segment spanning the *mchA* gene and the so called silent region was elucidated (Fig. 1b). Open reading frames (ORFs) adequately preceded by a possible ribosome binding site were searched for in both directions. From left to right, there was a 372-codon ORF that corresponded to the *mchA* gene. In the opposite direction, four ORFs were found, which were designated *mchS1* (421 codons), *mchS2* (77 codons), *mchS3* (144 codons), and *mchS4* (81 codons); all of them were located in the silent region (Fig. 1b).

The deduced amino acid sequences for the ORFs found were compared with sequences in data banks by using the program BLAST (1). MchA shared strong homology with proteins of the bacterial glycosyltransferase family, particularly with the subgroup of IroB proteins. IroB homologues belonging to the microcin M, H, and E gene clusters (accession numbers Q83ZA2, Q83ZB1, and Q93GK9) exhibited high levels of identity (99 to 85%). Following the IroB group, glycosyltransferases from polyketide-producing actinomycetes exhibited up to 34% identity with the MchA sequence. MchS1 was related to the ferric enterobactin esterase family, showing more than 50% identity with members from the subgroup of IroD proteins. The highest levels of identity (98 to 74%) were again exhibited by IroD homologues from the microcin M, H, and E gene clusters (accession numbers Q83ZA1, Q83ZB0, and Q93GK8). In addition, MchS1 shared a maximum of 27% identity with members of the Fes subgroup of enterobactin esterases. The deduced amino acid sequences for *mchS2*, *mchS3*, and *mchS4* did not show homologies with other proteins in data banks.

Strains carrying the MccH47 genetic system overproduce enterobactin. The ability of several strains to produce iron-

chelating activity was assayed in CAS plates as described in Materials and Methods. *E. coli* H47, as well as the MccH47-overproducing strain RYC1000(pEX100), gave rise to yellow halos of about 7 mm in diameter. RYC1000(pACYC184), which is isogenic to RYC1000(pEX100) but does not carry the antibiotic system, as well as RYC1000 and other *E. coli* K-12 laboratory strains, produced smaller halos of 4 to 5 mm in diameter, which we call basal CAS halos (Fig. 2). To analyze whether the MccH47 molecule could be responsible for the observed overproduction of iron-chelating activity, aliquots of an MccH47 extract were deposited on a CAS plate. No chelating activity was detected (data not shown).

Considering that enterobactin is the siderophore produced by all *E. coli* strains, we wondered if the large CAS halos seen with MccH47-producing strains corresponded to enterobactin overproduction. Therefore, UT400, a well-known enterobactin-deficient strain, was transformed with plasmid pEX100. The resulting clone did not produce any detectable iron-chelating activity. This phenotype reverted upon introduction of plasmid pENT1, which contains the entire *ent* system: the ability to produce CAS halos was recovered in strain UT400 *Mu*cts Ap^r(pENT1, pEX100) (Fig. 2). Thus, the overproduction of iron-chelating activity by strains carrying the MccH47 genetic system depended on the presence of the *ent* gene cluster.

The genetic determinant of the MccH47 system that is responsible for the overproduction of iron-chelating activity was searched for by transforming an Ent⁺ strain with plasmids carrying different *mch* DNA segments. The resulting strains were assayed on CAS plates, and only those containing *mchS4* overproduced iron-chelating activity. Thus, pMVDS4, a plasmid carrying only this gene, was constructed (Fig. 1c). RYC1000(pMVDS4) generated CAS halos of about 11 mm in diameter, while UT400(pMVDS4) produced no halo (Fig. 2). These results indicate that the iron-chelating activity overproduced under the effect of *mchS4* promotion depended on the presence of the *ent* genetic system and, therefore, that it would correspond to the strong iron chelator enterobactin. Monocatecholate compounds also synthesized by the Ent pathway (DHB and DBS) would not contribute significantly to the CAS halos, since spots of a concentrated DHB solution (2 mg/ml) did not change the color of the CAS plates (data not shown).

MccH47-producing strains never reached the level of siderophore production determined by the sole presence of pMVDS4, indicating that this effect could be compensated for by another activity encoded by the MccH47 genetic system. Therefore, we searched for an *mch* determinant which could accomplish this function by transforming RYC1000(pMVDS4) with a set of plasmids bearing different portions of the MccH47 system. Only those clones containing *mchS1* exhibited markedly reduced CAS halos. Thus, plasmid pUYS1, carrying only *mchS1*, was constructed (Fig. 1c). RYC1000(pMVDS4, pUYS1) as well as RYC1000(pUYS1) gave rise to minimal CAS halos, even smaller than the basal ones. Since MchS1 would belong to the family of enterobactin esterases, similar experiments were performed with plasmid pUY44, carrying only the *fes* gene from the *ent* system, and a similar effect was observed (Fig. 2).

Considering that the expression of the *ent* genes is subjected to iron regulation (7), siderophore production was also ana-

FIG. 2. Production of iron-chelating activity. Stabs of the indicated strains were made on CAS plates. Halos correspond to iron-chelating activity secreted by the stabs. For each strain, the mean and standard deviation of halo diameters from six separate assays are indicated. The absence of CAS halos around the stabs is indicated with a minus.

lyzed under iron-rich conditions. Consistently, a drastic reduction of the iron-chelating activity produced by all strains assayed was observed: MccH47-producing strains such as H47 and RYC1000(pEX100) did not generate any CAS halo, while RYC1000(pMVDS4) still produced halos, although they were reduced to around half of their original diameter. The same strains were also assayed at lower iron concentrations, 1/4 and 1/10 of that previously used, and in all cases they generated CAS halos, which increased in size as the iron concentration decreased (data not shown).

Dependence of MccH47 production on the *ent* genetic system. The Ent-deficient strain carrying the MccH47 system, UT400(pEX100), was assayed for antibiotic production as described in Materials and Methods. Surprisingly, it did not produce growth inhibition halos, while the reference strain RYC1000(pEX100) generated halos of about 18 mm in diameter. In contrast, the Ent⁺ isogenic strain UT400 Mucts Ap^r(pEX100, pENT1) was proficient for MccH47 production, indicating that the presence of the *ent* system was required for antibiotic production (Fig. 3). It must be added that phage Mucts Ap^r does not affect the analysis, since UT400 Mucts Ap^r(pEX100) was also deficient in antibiotic production.

The failure of strain UT400(pEX100) to produce the antibiotic could be due either to a defect in MccH47 synthesis or to a defect in its secretion. Thus, these possibilities were analyzed. It is known that cells carrying the MccH47 genetic system with mutations affecting microcin secretion do not produce antibiotics halos but keep the activity inside: their intracellular extracts are able to inhibit the growth of sensitive cells (8). To analyze whether UT400(pEX100) had an MccH47 secretion defect, intracellular extracts from this strain were obtained as described in Materials and Methods, and aliquots were spotted on a lawn of an MccH47-sensitive strain. As a

control, the same procedure was applied to an MccH47 secretion mutant affected in the *mchF* gene (8). After incubation, the only growth inhibition halo observed corresponded to the spot from the *mchF* mutant; UT400(pEX100) did not produce any effect. Therefore, no intracellular MccH47 activity could be detected in strain UT400(pEX100), suggesting that antibiotic production could be affected in some step before secretion, i.e., during antibiotic synthesis. To investigate whether the *ent*-deficient genetic context impeded the synthesis of the MccH47 precursor MchB, UT400 was transformed with plasmid pMVD10, carrying the microcin structural gene *mchB*, and transformants were selected on LB plates supplemented with chloramphenicol. No clone grew, thus showing that cells had suffered the toxic effect of MchB. As a control, the MccH47 immune strain UT400(pUY69) was transformed with

FIG. 3. Production of MccH47. Patch testing on a minimal M63 glucose plate seeded with a lawn of RYC1000(pUC13) and stabbed with the strains indicated is shown. Growth inhibition halos correspond to MccH47 produced by the stabs. Means and standard deviations of halo diameters from six separate assays are indicated.

FIG. 4. MccH47 production by strains with different *ent* genetic contexts. The *ent* genetic system is shown at the top. On the left are shown the *ent* genetic contexts of MC4100 $\Delta(ompT-fepC)267$ (a), MC4100 $\Delta(ompT-fepC)267(pUY47)$ (b), and MC4100 $\Delta(ompT-fepC)267(pUY46)$ (c). On the right are shown their phenotypes of MccH47 production and siderophore production (Ent) when they also bear pEX100. B, BamHI; H, HindIII; P, PstI.

pMVD10, and in this case abundant clones grew. These results indicated that the *ent* genetic deficiency did not prevent *mchB* expression or the toxic effect of its product. Other antibiotic synthesis steps, i.e., those involved in the maturation of the molecule, would therefore depend on the integrity of the *ent* genetic system.

The enterobactin synthesis pathway is involved in MccH47 production. The presence of the *ent* system determines the production of the main siderophore enterobactin and of the related catecholate compounds DHB and DBS. In a first assay, MccH47 production by UT400(pEX100) was analyzed in the presence of the enterobactin precursor DHB, and no antibiotic production was observed. Thus, the possible requirement for enterobactin was studied. A strain proficient for DHB synthesis and deficient for enterobactin synthesis was constructed by introducing the $\Delta(ompT-fepC)267$ deletion from UT400 into MC4100. As expected, the resultant Ent[−] strain transformed with plasmid pEX100 did not produce MccH47 (Fig. 4a). The $\Delta(ompT-fepC)267$ deletion removes genes from the *ent* system that are devoted to different functions related to enterobactin: synthesis (*entD* and *entF*), uptake (*fepA*, *fepE*, and *fepC*), and hydrolysis (*fes*). The involvement of *fepA* in MccH47 production could be eliminated, since the *fepA*::Tn10 strain FGB102(pEX100) produced antibiotics halos of similar size as those generated by the reference strain RYC1000(pEX100). In a second assay, MC4100 $\Delta(ompT-fepC)267(pEX100)$ was transformed with plasmid pUY47, which bears the *entD*, *fepA*, *fes*, and *entF* genes. The resultant strain turned Ent⁺ and fully

produced MccH47, indicating that *fepE* and *fepC* are not required for MccH47 production (Fig. 4b). MC4100 $\Delta(ompT-fepC)267(pEX100)$ was then transformed with pUY46, carrying *entD*, *fepA*, and *fes*, and in this case cells did not produce siderophore or MccH47 (Fig. 4c). These results showed that the enterobactin synthase EntF is necessary for MccH47 production.

Since the amount of siderophore produced and excreted by MccH47-producing strains depended on iron availability, we wondered whether MccH47 production could be dependent on iron availability as well. Several MccH47-producing strains, such as H47 and RYC1000(pEX100), were assayed for antibiotic production in minimal medium plates with increasing concentrations of iron (0, 1, 2.5, and 10 μg of $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ per ml). It should be taken into account that under iron-rich conditions, genes for the MccH47 receptors Cir, Fiu, and FepA are repressed and thus the indicator strain is less sensitive to the antibiotic (7, 24). Unexpectedly, the antibiotics halos were of similar size under all conditions; i.e., iron availability had no significant effect on MccH47 production (data not shown).

DISCUSSION

In this work we present results that relate the synthesis of a gene-encoded peptide antibiotic, MccH47, to the synthesis of a catecholate siderophore, enterobactin. Four lines of evidence support this relationship: (i) the promotion of enterobactin production exerted by the presence of the MccH47 genetic

system; (ii) the involvement of the enterobactin synthesis pathway in the production of MccH47; (iii) the presence in the MccH47 system of *iroB* and *iroD* homologues, relating the production of MccH47 to that of the catecholate siderophores salmochelins; and (iv) the previous finding that MccH47 uptake is mediated by any of the catechol receptors Cir, Fiu, and FepA (24).

By means of genetic analyses, *entF*, the gene encoding the enterobactin nonribosomal peptide synthase that finally assembles enterobactin (5), was demonstrated to be required for MccH47 production. In fact, whenever a strain was able to synthesize enterobactin, it proved to be proficient for MccH47 production when transformed with plasmid pEX100. Conversely, the Ent[−] strains analyzed did not produce the antibiotic even in the presence of the enterobactin precursor DHB. Although the possible requirement of other enzymes from the enterobactin synthesis pathway has not yet been analyzed, our results suggest that enterobactin, the main product of this pathway, would be necessary to achieve MccH47 production at some stage.

The stage of antibiotic production where the Ent pathway would participate was analyzed. MccH47 production is conceived as a process involving three main steps: synthesis of the precursor peptide MchB, subsequent maturation of the molecule, and its final secretion (20). In an Ent-deficient background, the inability to produce the antibiotic could not be related to any defect in the synthesis of MchB or in MccH47 secretion, thus pointing to MccH47 maturation as the stage where the contribution of the Ent pathway would be required. MccH47 maturation, in which *mchA*, *-C*, and *-D* gene products are known to be necessary, was proposed to endow the antibiotic molecule with the ability to enter cells (20). The additional participation of the Ent synthesis pathway in this process could well provide a catechol or a catechol-derived moiety for microcin synthesis. Thus, the mature molecule would be able to interact with the catechol receptors present on susceptible cells.

Two of the MccH47 receptors, Cir and Fiu, were shown to be involved in the transport of monomeric catechols and of analogous noncatecholate groups, but they do not operate in enterobactin uptake (10, 16). For this reason, enterobactin, a cyclic tricatecholate structure, might not be the final molecule that the Ent pathway would provide for MccH47 synthesis; its breakdown product DBS appeared to be a better candidate for that purpose. Fes is the enterobactin esterase encoded by the *ent* system that hydrolyzes ferric-enterobactin to liberate iron while producing DBS monomers, dimers, or linear trimers (7). In this sense, the presence in the MccH47 system of the *mchS1* gene, encoding a putative enterobactin esterase of the IroD subgroup, could be significant. Beyond the sequence similarity of IroD proteins to well-known ferric-enterobactin esterases, no specific function has yet been assigned to these proteins. In this work, a first indication about MchS1 activity is presented: the introduction of *mchS1* at a high gene dosage in an enterobactin-overproducing strain caused a marked reduction in the amount of extracellular siderophore. A similar effect was observed when *fes* was employed instead of *mchS1*. These results suggest that both gene products could work in a similar manner, i.e., that MchS1 could also hydrolyze enterobactin, and that both Fes and MchS1 would be able to break down the

molecule prior to its secretion. Therefore, it could be considered that MchS1 contributes to MccH47 synthesis by hydrolyzing enterobactin to provide enough DBS. Although *mchS1* null mutants still produce MccH47 (8), MchS1 participation in antibiotic synthesis cannot be absolutely discarded: these assays were always performed in a *fes*⁺ genetic context where *fes* could eventually complement the *mchS1* deficiency for MccH47 production. The nonviability of well-defined null *fes* mutants in *E. coli* impeded the testing of this possibility (our unpublished results).

The presence in the MccH47 genetic system of the *iroB* and *iroD* homologues *mchA* and *mchS1* raised the question of the possible relationship between MccH47 production and that of salmochelins. Moreover, it has been reported that *E. coli* CA46, which produces salmochelins, also produces microcins M and MccH47 (11). As mentioned above, MchS1 participation in MccH47 biosynthesis, although suspected, could not be confirmed. In contrast, MchA, a putative glycosyltransferase of the IroB subgroup, is clearly required (8). Considering that IroB from *S. enterica* would glycosylate DBS for the synthesis of salmochelins (11), we presume that MchA might accomplish a similar function: it may glycosylate DBS to provide a molecule, probably related to salmochelins, that would be employed in the construction of mature MccH47.

The requirement of enterobactin or, presumably, of a derivative salmochelin-type molecule for MccH47 synthesis would give meaning to the promotion of enterobactin production exerted by the presence of the MccH47 genetic system. The increased amounts of enterobactin would thus supply both functions, iron chelation and MccH47 synthesis. In the antibiotic system, *mchS4* was identified as the single gene responsible for enterobactin overproduction. Its effect partially persisted under iron-rich conditions, indicating that iron-mediated repression of siderophore production was overcome to an important extent by the action of MchS4. This could explain the fact that no decrease in MccH47 production was observed when iron was added to the medium. A possible explanation could be that MchS4 ensures the level of antibiotic produced under varied iron availability conditions by promoting the synthesis of sufficient enterobactin. The way that MchS4 performs this action remains unknown.

Based on these results and considerations, we outline a possible strategy that bacteria could use to synthesize MccH47. On one hand, microcin precursor is ribosomally synthesized as a 75-residue peptide, MchB. On the other hand, MchS4 enhances enterobactin synthesis, and some of the molecules produced are broken down into their building blocks of DBS by ester linkage hydrolysis performed by MchS1 and Fes. This hydrolysis could take place even before enterobactin is secreted. The DBS produced is glycosylated by the action of MchA, thus converting into a salmochelin-type molecule. The peptide precursor MchB, already possessing the ability to exert the specific deleterious action of MccH47, must then join glycosylated DBS or a derivative molecule to gain the ability to enter cells through the catecholate receptor proteins Cir, Fiu, and FepA. The *mchC* and *mchD* gene products, which are known to be necessary for MccH47 maturation, could be involved in the latter process.

MccH47 synthesis would thus appear to be a new strategy for making an antibiotic by combining the ribosomal synthesis of a

peptide with the thio template enzymatic synthesis of a catechol or catechol-derived compound, with both moieties together ensuring the antibiotic mechanism of action on susceptible cells. Other microcins could be synthesized in a similar dual way. In particular, microcin E492 appears to be related to MccH47 as judged by the striking similarities between their genetic systems, which include the presence of *iroBD* homologues (13). Although the microcin E492 peptide sequence and specific mode of action are unrelated to those of MccH47, microcin E492 could also benefit from the same "catechol strategy" for its synthesis. Indeed, microcin E492 as well as the newly described microcin M have recently been reported to employ the catechol uptake pathway for entry into cells (17). Perhaps other peptide antibiotics that make use of this strategy to reach their targets in gram-negative cells are still to be found.

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