Contribution of Gene Amplification to Evolution of Increased Antibiotic Resistance in *Salmonella typhimurium*

Song Sun,* Otto G. Berg,[†] John R. Roth[‡] and Dan I. Andersson*¹

*Department of Medical Biochemistry and Microbiology, Uppsala University, S-75123 Uppsala, Sweden, [†]Department of Molecular Evolution, Uppsala University, S-75236 Uppsala, Sweden and [‡]Department of Microbiology, College of Biological Sciences, University of California, Davis, California 95616

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ABSTRACT

The use of β -lactam antibiotics has led to the evolution and global spread of a variety of resistance mechanisms, including β -lactamases, a group of enzymes that degrade the β -lactam ring. The evolution of increased β -lactam resistance was studied by exposing independent lineages of *Salmonella typhimurium* to progressive increases in cephalosporin concentration. Each lineage carried a β -lactamase gene (*bla*_{TEM-1}) that provided very low resistance. In most lineages, the initial response to selection was an amplification of the *bla*_{TEM-1} gene copy number. Amplification was followed in some lineages by mutations (*envZ*, *cpxA*, or *nmpC*) that reduced expression of the uptake functions, the OmpC, OmpD, and OmpF porins. The initial resistance provided by *bla*_{TEM-1} amplification allowed the population to expand sufficiently to realize rare secondary point mutations. Mathematical modeling showed that amplification often is likely to be the initial response because events that duplicate or further amplify a gene are much more frequent than point mutations. These models show the importance of the population size to appearance of later point mutations. Transient gene amplification is likely to be a common initial mechanism and an intermediate in stable adaptive improvement. If later point mutations (allowed by amplification) provide sufficient adaptive improvement, the amplification may be lost.

THE extensive use of β -lactam antibiotics has led to the evolution and spread of many chromosomal, plasmid-, and transposon-borne resistance mechanisms (LIVERMORE 1995; WELDHAGEN 2004). Prominent among these mechanisms is a class of enzymes, β -lactamases, that hydrolyze the β -lactam ring (AMBLER 1980; POOLE 2004). TEM-1 β -lactamase, encoded by the *bla*_{TEM-1} gene, hydrolyzes both penicillins and early cephalosporins (MATAGNE *et al.* 1990). As bacteria developed resistance, stable extended-spectrum cephalosporins (ESCs) were introduced, leading to evolution of TEM sequence variants with improved ESC hydrolysis (PETROSINO *et al.* 1998). Resistance to β -lactams can also result from mutations that reduce levels of outer membrane proteins involved in uptake, altered target proteins (penicillin-binding proteins) to reduce β -lactam binding, or increased expression of efflux pumps that export the antibiotics (POOLE 2004; MARTÍNEZ-MARTÍNEZ 2008; ZAPUN *et al.* 2008).

Resistance to β -lactam antibiotics is linearly correlated with the lactamase level over a large range (NORDSTRÖM *et al.* 1972) and resistance to β -lactam

antibiotics can be provided by increasing enzyme levels. An early illustration of this process is the finding that *Escherichia coli* can develop ampicillin resistance by amplifying its *ampC* gene (EDLUND and NORMARK 1981). Similar amplification has been observed in both eubacteria and eukaryotes (CRAVEN and NEIDLE 2007; WONG *et al.* 2007) in response to various selective pressures, including antibiotics (ANDERSSON and HUGHES 2009; SANDEGREN and ANDERSSON 2009). In an unselected bacterial population, the frequency of cells with a duplication of any specific chromosomal region ranges between 10^{-2} and 10^{-5} depending on the region (ANDERSON and ROTH 1981), whereas a point mutation in that gene is expected to be carried by perhaps 1 cell in 10^7 – 10^8 (HUDSON *et al.* 2002). Thus, the rate of duplication formation is $\sim 10^{-5}$ /cell/division and further increases ~ 0.01 /cell/division (PETTERSSON *et al.* 2008) while the base substitution rate is $\sim 10^{-10}$ /cell/division/base pair (HUDSON *et al.* 2002). Thus, it is apparent that variants with an increased level of any enzyme activity are more likely to owe the increase to a gene copy number change than to a point mutation. Furthermore, because of the high intrinsic instability of tandem amplifications, haploid segregants are expected to take over the population when the selection pressure is released (PETTERSSON *et al.* 2008).

To examine the importance of gene amplification in bacterial adaptation to cephalosporins, several indepen-

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¹Corresponding author: Department of Medical Biochemistry and Microbiology, Uppsala University, S-75123 Uppsala, Sweden.
E-mail: dan.andersson@imbim.uu.se

FIGURE 1.—The region containing the *bla*_{TEM-1} gene. The structure of F'₁₂₈ was previously described (KOFOID *et al.* 2003).

dent *Salmonella typhimurium* lineages carrying the *bla*_{TEM-1} gene were allowed to develop resistance to progressively increased concentrations of cephalothin (a first-generation cephalosporin) and cefaclor (a second-generation cephalosporin). As these lineages developed resistance to higher antibiotic levels, amplification of the *bla*_{TEM-1} gene was the primary and most common resistance mechanism, which in some cases was followed by acquisition of rare point mutations that provided stable resistance.

MATERIALS AND METHODS

Strains and strain construction: Strains are all derivatives of *S. enterica* Var. Typhimurium LT2 (designated *S. typhimurium* throughout the text). The parental strain used for the evolution experiment DA11049 [*metA22*, *metE557*, *trpD*, *ilv-452*, *leu*⁻, *pro* (leaky), *hsdLT6*, *nsdSA29*, *hsdB*, *strA120*] carries an F'₁₂₈ plasmid with an engineered Tn10 transposon, designated transposon-β-lactamase and GFP (T-BAG), inserted in the *mhpC* gene (Figure 1). T-BAG was made by inserting an *rpsM-gfp-bla*_{TEM-1} construct into the mini-Tn10tet transposon on F'₁₂₈ by linear transformation (DATSENKO and WANNER 2000). An *rpsM-gfp-cat* construct was amplified by PCR from plasmid pZEP16 (HAUTEFORT *et al.* 2003), using oligonucleotides with 50 bp homology to the sequences located at ~2.4 kb on the Tn10tet transposon (length 2.9 kb). Then it was inserted into a Tn10tet transposon in a strain expressing Red recombination functions of phage lambda from plasmid pKD46. The T-BAG construct was completed by selectively adding the chromosomal *bla*_{TEM-1} gene from plasmid pUC19 (YANISCH-PERRON *et al.* 1985) in place of the plasmid *cat* gene by linear transformation. Oligonucleotides used for T-BAG construction, PCR, and identification of transposon insertion points and duplication join points are listed in the supporting information, Table S1.

Luria-Delbruck fluctuation test: From each of 10 independent cultures of the tester strain containing the *bla*_{TEM-1} gene (DA11049) and an isogenic strain lacking the *bla*_{TEM-1} gene (DA10057), 10⁶ cells were plated on plates containing 3 mg/liter of cefaclor. The number of colonies appearing after 24 hr

of incubation was determined and the mutation rate was calculated by the median method as previously described (LEA 1949). The distribution of mutants fitted a Luria-Delbruck distribution (not shown).

Experimental evolution of the T-BAG-carrying strain DA11049 in the presence of cephalothin and cefaclor: Strain DA11049 was grown overnight in LB medium without antibiotic and plated at several dilutions (from 10³ to 10⁵ cells/plate) on Luria agar (LA) plates containing increasing concentrations of antibiotic. After 1 day of incubation at 37°, two to five random colonies nearest a specific labeled region of the plate were picked irrespective of size, color, and appearance. The cells from each of the picked colonies were inoculated into 10 ml LB broth (containing the same concentration of cephalosporins as in the selection plates) and grown to full density at 37°. The fraction of plated cells that could grow on the increasing concentrations of cephalosporins was typically in the range of 1/10³–1/10⁴. From each of the cultures in the final cycle, 10³–10⁵ cells were spread on agar plates with an increased cephalosporin concentration (see Figure 2). From each passage 5 ml of culture were used for DNA isolation and 1 ml was mixed with 100 μl DMSO and stored at –80°. This was repeated for several cycles, generating multiple lineages of evolved bacteria, each with increasing resistance (see Table 1 and Figure 2).

Determination of minimum inhibitory concentration: The minimum inhibitory concentrations (MICs) of the different cephalosporins for all tested strains in this work were measured using *E*-tests from AB Biodisk as described by the manufacturer.

Isolation of RNA and DNA: Approximately 10 μl of frozen culture from each of the clones examined were inoculated into 10 ml LB broth containing the same concentration of cephalosporin as in the plate from which the clone was picked. The culture was grown at 37° until OD₆₀₀ values were 0.5 and total RNA, using the SV Total RNA Isolation System (Promega, Madison, WI), and DNA, using QIAGEN (Valencia, CA) Genomic-tip 100/G, were isolated from the same culture. RNA was treated with DNase I according to the user manual. Total RNA was also isolated from the three clones with only chromosomal point mutations (DA13407, DA13421, and DA13513) in the same way as described above, except that the culture was grown without cephalosporin present (since these mutations are stable, continued selection was not needed during growth).

FIGURE 2.—Phylogeny of clones selected for increased resistance to cephalosporins. The cephalosporin concentration (milligrams per liter) used at each selection step is indicated in parentheses following the strain number. (A) CE selection with cephalothin. (B) CF selection with cefaclor. Boxes indicate clones that were examined in detail with respect to DNA copy number, RNA levels, and MIC (Figure 3) as well as associated mutations (Table 3). The lineage number is indicated in italics at the endpoint strain for each lineage examined.

First-strand synthesis of cDNA from mRNA and quantitative real-time PCR: The mRNA was converted to cDNA using the cDNA reverse transcription kit from Applied Biosystems (Foster City, CA) according to the manufacturer's suggestions. The quantitative real-time PCR technique based on the high affinity of SYBR Green dye for double-stranded DNA was used. The fluorescence signal was monitored on-line, using the MiniOpticon real-time PCR system (Bio-Rad, Hercules, CA). The PCR amplification was performed by mixing 10 μ l diluted DNA or cDNA with 15 μ l mixture of primers and SYBR Green SuperMix (Bio-Rad). The *recA* gene was used as an internal control for all gene copy-number determinations. The DNA or mRNA levels of the *bla*_{TEM-1} gene were determined by using the standard curve method, and in every individual real-time PCR measurement the DNA or mRNA of the parental strain (DA11049) was used to normalize the DNA or mRNA fold change for the evolved strains. Oligonucleotides used for real-time PCR are listed in Table S1.

Isolation of Tn10dcam transposon linked to the cephalosporin-resistance mutations: A phage P22 HT lysate grown on a pool of randomly inserted Tn10dcam insertions in a wild-type genetic background was prepared and used to infect the cephalosporin-resistant strains. Chloramphenicol-resistant transductants were selected on LA plates supplemented with 30 mg/liter of chloramphenicol and by replica printing to LA plates with and without cephalosporin, we screened for loss of high-level cephalosporin resistance. Chloramphenicol-resistant and cephalosporin-susceptible clones were identified, and linkage of the chloramphenicol resistance marker (*i.e.*, Tn10dcam) to the cephalosporin resistance mutation was determined by backcrosses to the cephalosporin-resistant strain.

Identification of transposon insertion points: Transposon insertion points were identified using arbitrarily primed PCRs

in two steps. In the first reaction, transposon-specific oligonucleotides were mixed with arbitrary oligonucleotides consisting of a defined part and a variable part. An initial denaturation step of 5 min at 95° was followed by five cycles of 30 sec at 95°, 30 sec at 30°, and 1 min at 72° and then by another 30 cycles of 30 sec at 95°, 30 sec at 55°, and 1 min at 72°. The last elongation step was prolonged to 6 min. A second nested PCR was performed using 1 μ l of the first reaction mixture as a template. After 5 min of denaturation at 95°, 29 cycles of 30 sec at 95°, 30 sec at 54°, and 1 min at 72° were run, with the last elongation extended to 6 min. The resulting PCR fragments were purified and sequenced using an oligonucleotide with homology to the transposon (universal oligonucleotide). To identify the resistance mutations, genes in the vicinity of the transposon insertion were PCR amplified and sequenced with oligonucleotides that were designed on the basis of genome sequences of *S. typhimurium* LT2.

Identification of duplication join points: To determine the size of the amplified units and the structure of the duplication junctions, PCRs with mixes of oligonucleotides facing outward and at varying distance from the *bla*_{TEM-1} gene were performed. A PCR product is produced only if the reverse and forward oligonucleotides are brought to face each other over a duplication junction. PCR products unique for the strains carrying the amplifications were sequenced and the join points identified.

RESULTS

A parent strain whose *bla*_{TEM-1} gene confers low-level resistance to cephalosporins: To detect *bla*_{TEM-1} gene number while selecting for increased resistance, a hybrid operon was constructed in which both the

TABLE 1
Characteristics of strains isolated during *in vitro* selection for increased cephalothin or cefaclor resistance

Strains	Antibiotic and lineage	<i>bla</i> _{TEM-1}	Associated mutation	Selection concentration (mg/liter)	
				Cephalothin	Cefaclor
DA11049	Parental strain	Nonamplified	No	3	1.5
DA13388	CE lineages 1–4	Amplified (<i>n</i> = 2)	No	8	
DA13390	CE lineages 5 and 6	Amplified (<i>n</i> = 2)	No	12	
DA13392		Amplified (<i>n</i> = 2)	No	12	
DA13394	CE lineages 1–4	Amplified (<i>n</i> = 3)	No	16	
DA13396		Amplified (<i>n</i> = 2)	No	12	
DA13398	CE lineage 5	Amplified (<i>n</i> = 2)	No	48	
DA13400	CE lineage 6	Amplified (<i>n</i> = 3)	No	24	
DA13407	CE lineage 4	Amplified (<i>n</i> = 2)	<i>envZ</i> (GGC → GAC, G226D)	48	
DA13409	CE lineages 1–3	Amplified (<i>n</i> = 5)	No	48	
DA13413		Nonamplified		20	
DA13415		Nonamplified		20	
DA13417	CE lineage 5	Nonamplified		256	
DA13418		Nonamplified		32	
DA13421	CE lineage 6	Nonamplified	<i>cpxA</i> (CCG → CAG, P131Q)	>256	
DA13423		Nonamplified		32	
DA13425		Nonamplified		32	
DA13436	CE lineage 3	Amplified (<i>n</i> = 15)	No	96	
DA13438		Amplified (<i>n</i> = 7)	No	48	
DA13442	CE lineage 1	Amplified (<i>n</i> = 14)	No	96	
DA13443	CE lineage 2	Amplified (<i>n</i> = 24)	No	>256	
DA13444		Amplified (<i>n</i> = 10)	No	52	
DA13445		Amplified (<i>n</i> = 10)	No	40	
DA13446		Nonamplified		40	
DA13448		Amplified (<i>n</i> = 4)	No	40	
DA13449		Nonamplified		40	
DA13450		Amplified (<i>n</i> = 7)	No	40	
DA13451	CE lineage 3	Amplified (<i>n</i> = 39)	No	>256	
DA13452		Amplified (<i>n</i> = 19)	No	200	
DA13453		Amplified (<i>n</i> = 26)	No	200	
DA13454		Amplified (<i>n</i> = 27)	No	200	
DA13457		Amplified (<i>n</i> = 15)	No	200	
DA13458		Amplified (<i>n</i> = 10)	No	200	
DA13459		Amplified (<i>n</i> = 16)	No	200	
DA13460		Amplified (<i>n</i> = 7)	No	200	
DA13478		Nonamplified			2
DA13481	CF lineages 7 and 8	Amplified (<i>n</i> = 2)	No		3
DA13482		Amplified (<i>n</i> = 2)	No		8
DA13484		Amplified (<i>n</i> = 2)	No		8
DA13487	CF lineage 8	Amplified (<i>n</i> = 2)	No		4
DA13489	CF lineage 7	Amplified (<i>n</i> = 2)	No		6
DA13491		Nonamplified			22
DA13493		Nonamplified			22
DA13501		Amplified (<i>n</i> = 2)	No		22
DA13503		Amplified (<i>n</i> = 3)	No		22
DA13511		Nonamplified			22
DA13513	CF lineage 8	Nonamplified	<i>nmpC</i> (G deletion at bp 680 in the coding sequence)		12
DA13516		Amplified (<i>n</i> = 2)	No		22
DA13518	CF lineage 7	Amplified (<i>n</i> = 3)	No		10
DA13520		Nonamplified			22

*bla*_{TEM-1} and the *gfp* gene (encoding GFP) are expressed from a single constitutive promoter. This operon was placed on an F₁₂₈ plasmid such that any resistance mutations affecting either *bla*_{TEM-1} copy number or the *bla*_{TEM-1} sequence could easily be identified by its conjugative transferability and distinguished from any mutation in the chromosome. Using this tester strain (DA11049), any bacterial colony whose increased antibiotic resistance was due to *bla*_{TEM-1} amplification could be inferred by its green color (reflecting increased *gfp* expression). Any resistance due to chromosomal mutations was demonstrable by its failure to be co-inherited when the plasmid was conjugally transferred to a new recipient strain.

To identify cephalosporins for use in the selection experiments, the tester strain (DA11049) and an isogenic strain lacking the *bla*_{TEM-1} gene (DA10057) were examined for their resistance to seven different cephalosporins (Table 2). With two of these cephalosporins, cephalothin and cefaclor, the tester showed a small but reproducibly higher MIC (1.5- to 2-fold) than the control strain, suggesting that the TEM-1 β -lactamase provided a very low cephalosporinase activity against these antibiotics.

Mutants with increased cephalosporin resistance appear rapidly: To examine if presence of the *bla*_{TEM-1} gene influenced the apparent mutation rate to increased cephalosporin resistance we performed a Luria–Delbruck fluctuation test. We observed an ~10-fold higher mutation rate in the strain with the *bla*_{TEM-1} gene as compared to the strain without the *bla*_{TEM-1} gene (1.6×10^{-3} vs. 1.5×10^{-4}). This very high apparent mutation rate probably reflects the existence of a number of different mechanisms that can increase resistance 2- to 3-fold (including the mechanisms described below). To try to understand how presence of the *bla*_{TEM-1} gene could contribute to the increase in apparent mutation rate, we examined how improved resistance to cephalosporins evolved by plating independent lineages of the tester strain (DA11049) on a succession of agar plates containing increasing levels of antibiotic. Figure 2 shows the phylogeny of the evolved lineages, where a DA number indicates a specific clone that was isolated at a specific selection step. After only three passages, resistance to cefaclor increased from an MIC of 1.5 mg/liter to 22 mg/liter; after five passages, resistance to cephalothin increased from an MIC of 3 mg/liter to 200 mg/liter. Some of the mutant colonies had a green color (not shown), suggesting that the level of expression of the *gfp* gene had increased as well.

Cephalosporin resistance improved via multiple pathways of adaptation: Gene duplications are expected to occur at a frequency several orders of magnitude greater than any point mutation that might generate a stronger promoter or increase the specific activity of an enzyme (ANDERSON and ROTH 1981). Therefore it seemed likely that mutants with increased

TABLE 2
Low-level resistance to cephalosporins conferred by the TEM-1 β -lactamase

Antibiotic	MIC (mg/liter)	
	DA10057 TEM-1 absent	DA11049 TEM-1 present
Cefuroxime	2	2
Cefpirome	0.047	0.047
Cefpodoxime	0.25	0.25
Cephalothin ^a	2	3
Cefepime	0.032	0.032
Cefotaxime	0.064	0.064
Cefaclor ^a	1	1.5

^a Chosen for selection experiments.

resistance might show an amplification of the entire region including the parental *bla*_{TEM-1} gene. In an initial screening, all 51 clones isolated after different numbers of passages (Table 1 and Figure 2) were examined by real-time PCR and agar diffusion of antibiotics (*E*-tests) to determine *bla*_{TEM-1} copy number and MIC. All 51 clones showed an increased MIC and 70% of the clones (36/51) showed an increased *bla*_{TEM-1} copy number, demonstrating that gene amplification was the most common response to selection.

We examined in detail the genetic characteristics of clones isolated sequentially from six lineages evolving with cephalothin (boxed clones in Figure 2A) and from two lineages evolving with cefaclor (boxed clones in Figure 2B). These clones were chosen to represent lineages with different MICs and level of gene amplification. DNA and RNA levels of the *bla*_{TEM-1} gene were measured by real-time PCR and the MICs were determined using *E*-tests. Of the eight lineages examined, four had a similar evolutionary trajectory in which the increase in MIC was paralleled by an increase in *bla*_{TEM-1} RNA level and *bla*_{TEM-1} DNA amplification (Figure 3A). The other four lineages showed a pattern in which initial MIC increase was accompanied by an increase in the DNA and RNA levels, but subsequent MIC increase occurred with either constant or reduced levels of the DNA and RNA levels (Figure 3B). In some cases, the initial copy number remained elevated [*e.g.*, cephalothin (CE) lineage 4 and cefaclor (CF) lineage 8]. In other cases, the initial amplification segregated to the haploid state (*e.g.*, CE lineages 5 and 6). The latter trajectories suggested that the initial amplification response was superseded by later mutations that provided increased resistance by a mechanism that did not require *bla*_{TEM-1} gene amplification, thereby relaxing selection on the amplification and allowing its loss by segregation. To the extent that these trajectories improved resistance without an increase in *bla*_{TEM-1} RNA level, it would appear that the later mutations did not involve changes in control of *bla*_{TEM-1} gene expression. These secondary mutations were identified and characterized.

FIGURE 3.—Six lineages from the CE and two lineages from the CF evolution experiments were chosen to examine changes in DNA and RNA levels of the *bla*_{TEM-1} gene and MIC values for all isolated clones during evolution in the presence of increasing levels of cephalothin or cefaclor. (A) Lineages with parallel increases in MIC and *bla*_{TEM-1} gene copy number. (B) Lineages with increased MIC, initial *bla*_{TEM-1} gene amplification, and subsequent secondary resistance mutations. The standard deviation of relative fold change in DNA and RNA levels is ~15%.

Stable mutations that confer cephalosporin resistance: To identify the late mutations that conferred resistance without continued amplification, we examined three independent resistant mutants, all of which showed an initial increase in copy number. One mutant was from the cefaclor-resistant lineage 8 (DA13513) in which *bla*_{TEM-1} copy number dropped very little over time. The second was from the cephalothin-resistant lineage 4 (DA13407) in which copy number remained constant. The third mutant was from the cephalothin-resistant lineage 6 (DA13421) that showed initial amplification and subsequent segregation (see Figures 2 and 3).

First, the *bla*_{TEM-1} gene and the *rpsM* promoter region were sequenced from all four mutants and revealed no changes that could account for the increased expression of the *bla*_{TEM-1} and *gfp* genes. Second, to identify the secondary resistance mutations inferred to be unassociated with the *bla*_{TEM-1} gene, we sought chromosomal Tn10 insertions linked to the increased resistance in the three cephalosporin-resistant strains DA13407, DA13421, and DA13513 as described in MATERIALS AND METHODS. The chromosomal location of these linked Tn10dcaM insertions was determined by sequencing the junction fragments following semiran-

dom PCR (MATERIALS AND METHODS). The DNA base sequence of these fragments revealed the chromosomal site of the Tn10dcam insertion. From the determined insertion point and the transduction linkage of the resistance mutations to the transposon (data not shown), the candidate genes conferring cephalosporin resistance were mapped and the sequence change was identified in the affected chromosomal region.

Strain DA13407 (CE lineage 4) (in which initial *bla*_{TEM-1} expression dropped very little) acquired a missense mutation in the *envZ* gene, the sensor part of the *envZ-ompR* two-component system (TCS) that regulates expression of the outer membrane porins OmpF and OmpC (CAI and INOUE 2002; NIKAIIDO 2003). Thus this mutation is expected to reduce expression of two porins. Strain DA13421 (CE lineage 6) (in which *bla*_{TEM-1} gene copy number dropped) carried a missense mutation in the *cpxA* gene, the sensor part of another two-component system that regulates expression of OmpF and OmpC (BATCHELOR *et al.* 2005). Finally, strain DA13513 (CF lineage 8) (in which *bla*_{TEM-1} copy number decreased slightly) carried a frameshift mutation in the *nmpC* gene, encoding the porin OmpD (NIKAIDO and VAARA 1985; SINGH *et al.* 1996).

To confirm that these point mutations arose after *bla*_{TEM-1} gene amplification, the *envZ*, *cpxA*, and *nmpC* genes were sequenced from earlier intermediates in these lineages (Figure 3, A and B; CE lineage 4, strains DA13388 and DA13394; CE lineage 6, strains DA13390 and DA13400; and CF lineage 2, strain DA13481). In these earlier *bla*_{TEM-1} gene amplification clones, no *envZ*, *cpxA*, or *nmpC* mutations were found, demonstrating that the initial increase in resistance was due to *bla*_{TEM-1} amplification and strains with this amplification later acquired mutations in the *envZ*, *cpxA*, or *nmpC* genes.

Expression levels of the porin genes in *envZ* and *cpxA* mutants: The expression levels of the mRNAs encoding three major porin proteins were determined for the *envZ* (DA13407) and *cpxA* (DA13421) mutants and for the parental strain used for the cephalosporin selection (DA11049) (Figure 4). In the *envZ* mutant (DA13407; slight drop in *bla*_{TEM-1} copy number), expression of the *ompC* and *nmpC* genes was completely turned off (>100-fold reduction) and expression of *ompF* was reduced 5-fold. Similarly, in the *cpxA* mutant (DA13421; lost *bla*_{TEM-1} amplification), expression of *ompC*, *ompF*, and *nmpC* was reduced 50-, 2-, and 30-fold, respectively. These results showed that the mutations identified in the two sensor kinases of the EnvZ-OmpR and CpxA-CpxR TCSs downregulated expression of the porin mRNAs. This downregulation most likely increased the cephalosporin resistance by decreasing porin levels and thereby reducing permeability of the outer membrane to cephalosporins (OPPEZZO *et al.* 1991; NIKAIIDO 2003; MARTÍNEZ-MARTÍNEZ 2008).

FIGURE 4.—mRNA levels of the genes encoding the porin proteins (OmpC, OmpF, and OmpD) in *cpxA* (DA13421) and *envZ* (DA13407) mutants and wild type (DA11049). The relative fold change for each *omp* mRNA was calculated as the expression level of the cephalosporin-resistant mutants divided by that of the parental T-BAG strain (DA11049). The standard deviation of relative fold change in mRNA levels is ~10%.

Evidence that resistance provided by later point mutations is independent of the preceding *bla*_{TEM-1} gene amplification: To determine whether the effect of stable resistance mutations *ompC*, *ompF*, and *nmpC* depends on the preceding amplification of *bla*_{TEM-1}, each mutation was placed in two different genetic contexts—one with a *bla*_{TEM-1} deletion and one with an unamplified *bla*_{TEM-1} gene. These strains were tested to determine their MIC of a series of cephalosporins (Table 3). In strains with an unamplified *bla*_{TEM-1} gene, all three mutations caused high-level resistance to all tested cephalosporins (in Table 3, compare lines 4–6 to the parent in line 1) whereas in strains lacking a *bla*_{TEM-1} gene, the *cpxA* and *nmpC* mutations caused only modest increases in MIC values and the *envZ* mutation had an effect only for two of the antibiotics (in Table 3, compare lines 4–6 with 7–9). Thus mutations that reduced porin levels caused high-level resistance only in strains possessing a *bla*_{TEM-1} gene, showing that the amplification facilitated selection of stable mutations with high-level resistance, but was not essential to their resistance phenotype.

Size and endpoints of amplified units: The amplified units were characterized for five clones with *bla*_{TEM-1} gene amplifications. Analyzed strains from the CE evolution experiment had a 7-, a 39-, and a 4-fold *bla*_{TEM-1} amplification (DA13438, DA13451, and DA13448). Strains from the CF evolution experiment had a 2- and a 3-fold *bla*_{TEM-1} amplification (DA13487 and DA13518). Three of these five strains (DA13487, DA13518, and DA13448) amplified a region whose duplication formed by recombination between identical sequence repeats of IS3 that flanked the *bla* gene (the 1.26-kb IS3A and IS3C elements in Figure 5). In these three strains, a unit of 134 kbp was amplified to

TABLE 3
Susceptibility of evolved mutants to various cephalosporins

Strains	<i>bla</i> _{TEM-1}	Associated mutation	Lineage cycle	^a MIC (mg/liter)						
				CE	CF	CR	CT	PM	PX	XM
DA11049	Nonamplified	No	Lineage 1, cycle 0	3–4	1.5–2	0.047–0.064	0.047	0.032–0.047	0.19–0.25	2
DA13442	Amplified (<i>n</i> = 14)	No	Lineage 1, cycle 4	96	12	0.38	0.19	0.25	0.5	6
DA13451	Amplified (<i>n</i> = 39)	No	Lineage 3, cycle 5	>256	32	0.75	0.25	0.5	1	8
DA13407	Nonamplified	<i>envZ</i> (GGC → GAC, G226D)	Lineage 4, cycle 3	48	6	0.38	0.125	0.125	0.5	8
DA13513	Nonamplified	<i>nmpC</i> (one G deletion at bp 680 in the coding sequence)	Lineage 8, cycle 3	24	12	0.5	0.19	0.25	0.75	6
DA13421	Nonamplified	<i>cpxA</i> (CCG → CAG, P131Q)	Lineage 6, cycle 3	>256	48	0.38	0.5	0.19	4	>256
DA14556	None	<i>envZ</i> (GGC → GAC, G226D)		4	1.5	0.064	0.094	0.032	0.25	4
DA14478	None	<i>nmpC</i> (one G deletion at bp 680 in the coding sequence)		12	4	0.25	0.19	0.125	1	12
DA14473	None	<i>cpxA</i> (CCG → CAG, P131Q)		24	8	0.5	0.75	0.25	3	64

^aCE, cephalothin; CF, cefaclor; CR, cefpirome; CT, cefotaxime; PM, cefepime; PX, cefpodoxime; XM, cefuroxime.

copy numbers of 2, 3, and 4 (in DA13487, DA13518, and DA13448). The remaining two strains (DA13438 and DA13451) had an amplification that formed by recombination between 400 bp of imperfect homology shared by the Rep23 and Rep31 sequences. In these two strains a unit of 36 kbp was amplified to attain copy numbers of 7 and 39, respectively (Figure 5). These particular amplifications were previously observed during selection for reversion of a leaky *lac* mutation located on F₁₂₈ (KUGELBERG *et al.* 2006).

Mathematical modeling shows the conditions under which gene amplification can facilitate resistance development: In the experiments described above, *bla*_{TEM-1} amplification was the most frequent resistance mechanism found during selection on cephalosporin, but some resistant strains arose with no evidence of amplification. To estimate quantitatively the conditions under which gene amplification might be expected to dominate adaptive evolution and the magnitude of that dominance, we used estimated rates of the several event types and calculated the probability of developing resistance. The probability of developing initial resistance by gene amplification was compared to the probability of directly acquiring point mutations (see the APPENDIX and Figures 6 and 7).

In this analysis, experimentally estimated rates of duplication (3×10^{-4}) and further amplification (0.05) were used with estimated fitness costs ($s = -0.1$) to predict the distribution of gene copy number variants in an unselected population (PETERSSON *et al.* 2005, 2008) (Figure 6). The rate of point mutation was estimated for the types of mutations described here ($m = 10^{-8}$) and used to predict the frequency of resistant point mutants. The model suggests that in an unselected population of 10^8 cells the number of cells with a point mutation capable of providing resistance would be about the same as the fraction with a six-copy amplification (~10 cells of each type) (Figure 7). Thus if equivalent resistance could be provided by either a point mutation or an amplification of eight or more copies, one might expect about half of the resistant clones to rely on amplification. While this conservative calculation suggests a significant role for amplification, it vastly underestimates the real role of amplification.

It seems highly likely that a modest level of resistance is provided by the more frequent low-copy-number variants (such variants provide fewer copies of the resistance gene, but also have lower inherent fitness cost). Thus as soon as selection is applied, cells with smaller (but more frequent) amplifications can grow and expand subpopulations in which fitter variants with further amplification are frequent. This is made likely by the extremely high rates of copy number changes once more than one copy is present (10^{-2} /cell/generation). As long as some growth is possible, cells with any array of *bla*_{TEM-1} copies can very quickly expand that array to any

FIGURE 5.—Size and endpoints of amplified units in five clones with amplified bla_{TEM-1} genes. Copy number of the amplified unit is indicated with n . Numbering and designations of Rep sequences and IS3 elements were previously described (KOFOID *et al.* 2003).

size that is required to provide the necessary level of resistance (PETTERSSON *et al.* 2005).

In a smaller population, $N = 10^6$, point mutations are not likely to be present, while arrays of four or fewer are almost certain to be present initially (Figure 7). Under these conditions, amplified arrays will obviously predominate in causing increases in resistance. Thus, amplified arrays not only have an initial advantage over point mutations because they preexist at much higher frequency, they are also more adaptable and quicker to pick up further improvements, whenever increases in gene dosage can enhance resistance. The modeling therefore suggests that gene amplification will predominate over point mutations under most conditions, in particular when population sizes are small. Such amplification is likely to be intermediate in development of stable resistance because it allows a primary expansion of the population to a level that can support occurrence of rare point mutations. This intermediate role of amplification may be missed when stable intermediates

have been established either because the amplification is no longer needed or because the amplification is lost after selection is relaxed.

DISCUSSION

Experiments describe how cephalosporin resistance evolves in response to increasing concentration of drug. Tandem gene amplification of the bla_{TEM-1} gene was not only the predominant initial response to selection, but it also preceded development of genetically stable resistance caused by reduction of porin levels. The major conclusion in this work is that amplifications are the primary response to selection and enhance the opportunity for rarer subsequent stable resistance mutations by allowing growth and thereby increasing population size.

Three stable chromosomal mutations are described that appeared in strains with bla_{TEM-1} gene amplification. Two of these ($nmpC$ and $cpxAR$) have not been described previously. All three mutation types conferred

FIGURE 6.—Expected fraction of the population that carries an amplification containing i extra gene copies as a function of the s -value per copy for $i = 1, 2, 3, 5, 8$ (solid curves from top to bottom). Curves are drawn between the simulated data points as indicated. Dashed curves are for a point mutation, Equation A3, assuming $u = 10^{-7}, 10^{-8},$ or 10^{-9} , from top to bottom. $k_{dup} = 3 \times 10^{-4}$ and $k_{rec} = 0.05$ assumed in all cases.

FIGURE 7.—The probability from Equation A4 that the population has at least one individual carrying an array with i extra gene copies (solid curves with $i = 3, 4, 5, 8, 10$ from top to bottom) or at least one with a point mutation (dashed curves for $u = 10^{-7}, 10^{-8},$ and 10^{-9} , from top to bottom). The data from Figure 6 and $N = 10^6$ were used. The two dotted curves with open symbols show the results for $i = 4$ and $N = 10^7$ (top) or $N = 10^5$ (bottom).

resistance by reducing expression of one or more of the porins, OmpC, OmpF, or OmpD. Previous work showed that loss or modification of porins can confer resistance to β -lactams (NIKAIDO 2003) and that mutations in the *envZ* and *cpxA* genes may reduce the porin gene expression. The two-component system EnvZ–OmpR responds to osmolarity changes and phosphorylated OmpR modulates transcription of the genes for the outer membrane porin proteins OmpF and OmpC (CAI and INOUE 2002; NIKAIDO 2003). Similarly, the two-component system CpxA–CpxR senses envelope stress (DOREL *et al.* 2006) and CpxR can bind upstream of the promoters of the *ompF* and *ompC* genes to regulate their expression (BATCHELOR *et al.* 2005). Thus, mutations in the sensor proteins EnvZ and CpxA could reduce porin expression by reducing phosphorylation of the cognate regulators. Finally, the *nmpC* gene encodes the OmpD porin and a single-nucleotide frameshift mutation in this gene will inactivate the porin.

Two properties of gene amplifications are relevant to their role in resistance development—their formation frequency and instability. The high steady-state frequency of duplications (10^{-5} – 10^{-2} depending on region) in an unselected population provides a large reservoir of standing genetic variation (ANDERSON and ROTH 1981). From this reservoir of copy number variants, selective pressure can favor cells with increased copy number of any specific region. A second important characteristic of duplications is their intrinsic instability. Thus, if tandem gene amplifications are not continuously selected, they will be rapidly lost from the population, with mechanistic rates as high as 0.15/generation/cell (PETTERSSON *et al.* 2008). The inherent fitness cost of duplications might make the apparent loss rates even higher. The *bla*_{TEM-1} gene amplifications examined here show a loss in copy number that approaches the wild-type level within <50 generations of nonselective growth (data not shown).

There are broader medical implications of the presented experiments and modeling. Preexisting amplification of the β -lactamase gene should be expected whenever bacteria with a native *bla* gene are exposed to cephalosporins. Since all regions of the genome are subject to duplication and amplification, resistance to many antibiotics may evolve by processes that include amplification of some resident gene (BERTINI *et al.* 2007; BROCHET *et al.* 2008). It is of interest that plasmids carrying β -lactamase genes usually contain potential regions of homology for generating duplications (*e.g.*, IS elements), and therefore the frequency of plasmid-borne preexisting *bla* gene duplications in a population is expected to be very high. This is supported by studies that have shown how the gene copy number of various resistance genes is increased in mutants with increased resistance (HASHIMOTO and ROWND 1975; PERLMAN and STICKGOLD 1977; PETERSON and ROWND 1985; NICHOLS and GUAY 1989; HAMMOND *et al.* 2005; NICOLOFF *et al.* 2006, 2007).

Furthermore, as demonstrated by our modeling, cells with some duplication or low amplification in place can very quickly expand the array to any copy number that is required to provide the necessary level of resistance. The highest *bla*_{TEM-1} copy number observed in this experiment was 40. However, because of the instability and high loss rate of tandem amplification in the absence of selection, it is likely that resistance generated by gene amplification goes undetected in the clinical microbiology laboratory, resulting in an underestimation of the clinical significance of spontaneously acquired antibiotic resistance (CRAVEN and NEIDLE 2007).

The present findings suggest that gene amplification facilitates the process of acquiring stable adaptive mutations by increasing the number of selected target genes in the population. These stable changes can occur outside of the amplified region and be allowed by the population expansion alone (as described here) or can affect the amplified genes (ROTH *et al.* 2006), in which case target number is increased by both cell growth and added copies per cell. Since the parent amplification may be dispensable after a stable improvement occurs, it is conceivable that the intermediate amplifications leave no trace in the genome (ROTH *et al.* 2006). For example, the sequence evolution of TEM β -lactamases that has been observed in clinical settings (PETROSINO *et al.* 1998; MAJIDUDDIN and PALZKILL 2003) could have occurred in a sequential manner from (i) an initial low-activity single-copy gene with low resistance, (ii) amplification of the low-activity gene to increase resistance, (iii) occurrence of a point mutation in one gene copy to increase specific activity of β -lactamase, and (iv) segregation of the array with selective maintenance of the mutationally improved copy. It remains to be experimentally determined if and how rapidly the amplified *bla*_{TEM-1} genes can diverge and acquire different point mutations that can increase the specific activity against different cephalosporins. Such mutations were not observed in this experiment, probably because other unrelated resistance mutations (*i.e.*, *envZ*, *cpxA*, and *nmpC*) occurred at a higher rate and therefore predominated in the improvement process. This idea is supported by the fact that improved cephalosporinase activity of TEM-1 requires several sequential specific point mutations (HENQUELL *et al.* 1995; KNOX 1995; SIROT *et al.* 1997; ZACCOLO and GHERARDI 1999) and with the small population sizes used in our selection experiments they are very unlikely to have been isolated.

Finally, the process described here is applicable to any aspect of adaptive evolution in which increased activity of some gene can enhance growth. Thus, whenever growth is limited by an external factor (*e.g.*, low nutrient levels) or an internal factor (*e.g.*, a deleterious mutation), and the problem may be mitigated by increasing the levels of a gene product, common gene amplifications are likely to be selected. Evidence that this actually occurs is the frequent detections of gene amplification in bacteria

during growth on limited carbon sources (TLSTY *et al.* 1984; SONTI and ROTH 1989), in the presence of various toxic compounds (EDLUND and NORMARK 1981; FOGEL and WELCH 1982; GOTTESMAN *et al.* 1995; NEWCOMB *et al.* 2005), and in mutants with reduced fitness (NILSSON *et al.* 2006; PAULANDER *et al.* 2007).

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APPENDIX: MODELING OF THE PROBABILITY OF GENE AMPLIFICATION VS. POINT MUTATION

On the basis of a previously formulated model (PETTERSSON *et al.* 2005, 2008), we calculated the expected equilibrium distribution of amplifications in a population. The following parameters are needed: k_{dup} is the rate with which a new duplication occurs in the region of interest; k_{rec} is the rate of homologous recombination, once the first duplication is in place. These recombination events can lead to an increase or a decrease in copy number. s is the change in relative growth rate for an individual that carries a single duplication; in general, amplification is associated with a metabolic cost and $s < 0$. Although it can be argued that additional copies influence the growth rate in a nonlinear way, in the calculations below the fitness for an individual that carries i extra copies is assumed to be $(1 + s)^i$. In each replication, the probability for a recombination event that changes the number of extra copies from j to i is given by

$$p(i | j) = \frac{k_{\text{rec}} j}{1 + k_{\text{rec}} j} \cdot \frac{i + 1}{(j + 1)^2}, \text{ for } 0 \leq i \leq j \quad (\text{A1a})$$

and

$$p(i | j) = \frac{k_{\text{rec}} j}{1 + k_{\text{rec}} j} \cdot \frac{2j - i + 1}{(j + 1)^2}, \text{ for } j \leq i \leq 2j + 1. \quad (\text{A1b})$$

From simulations based on this model, we find the equilibrium distribution as the expected fractions of individuals that carry i extra gene copies, $f_i(s)$; see Figure 6. For a single duplication ($i = 1$), and as long as $f_1 \ll 1$, the result conforms to the intuitively reasonable expression

$$f_1(s) = \frac{k_{\text{dup}}}{k_{\text{rec}}/3 - s}. \quad (\text{A2})$$

The numerical factor 3 is approximately valid for $s = 0$ and decreases to ~ 2 for $s = -0.2$. A duplication appears with rate k_{dup} and is lost with rate $\sim k_{\text{rec}}/4$ (Equation A1a), although the overall loss rate is also influenced by the possibility to form larger arrays. The estimated values of k_{rec} vary between 0.015 and 0.15 and those of k_{dup} vary between 10^{-5} and 10^{-2} (PETTERSSON *et al.* 2008). In the numerical comparisons below, we assume $k_{\text{rec}} = 0.05$ and $k_{\text{dup}} = 3 \times 10^{-4}$ throughout as reasonable averages. Changes in k_{dup} translate directly to a proportional change in f_i . Equation A2 and can be compared to the expectation for the presence of a point mutation that appears with rate u and carries a cost corresponding to the fitness loss s (< 0). At equilibrium, such a mutation is expected to be present in a fraction of

$$f = -u/s \quad (\text{A3})$$

in the population. Individual point mutations appear with rates on the order of 10^{-10} per generation. If we consider a number of different point mutations that have similar effect, u could be on the order of 10^{-8} .

These fractions (Figure 6) give the expected equilibrium present in a very large population. Equilibrium, or more appropriately mutation–selection balance, is established rapidly under nonselective conditions when both k_{dup} and k_{rec} are large. Without specifying background history, equilibrium seems the most reasonable assumption. If the starting population (of size N) is considered as a sample from such an equilibrated large population, the probability that it contains at least one individual carrying an array of size i is

$$P_i = 1 - \exp(-Nf_i). \quad (\text{A4})$$

If $f_i > 1/N$, the initial presence of such an array is virtually assured. The same result holds for the point

mutation if f_i is replaced by f in Equation A4. If $N = 10^6$ and $s = -0.1$, the preexisting presence of arrays up to $i = 4$ is nearly certain, while the presence of individuals carrying a point mutation is uncertain (Figure 7). In fact, even for populations as small as $N = 10^3$, the presence of a preexisting duplication is nearly certain ($f_1 > 10^{-3}$ in Figure 6). For populations around $\geq 10^8$,

most of the variants considered in Figure 6 will be present with a probability near one ($f_i > 10^{-8}$), except possibly long arrays and rare point mutations with high fitness cost. We note that the probability of preexistence, Equation A4, is very sensitive to sample size. Since f_i is proportional to k_{dup} , it is equally sensitive to variations in k_{dup} .

GENETICS

Supporting Information

<http://www.genetics.org/cgi/content/full/genetics.109.103028/DC1>

Contribution of Gene Amplification to Evolution of Increased Antibiotic Resistance in *Salmonella typhimurium*

Song Sun, Otto G. Berg, John R. Roth and Dan I. Andersson

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TABLE S1

Oligonucleotides used for PCR, sequencing, real-time PCR, linear transformation and identification of duplication join points and transposon insertion points

Gene	Purpose	Oligonucleotide name	Sequence (5'-3')
<i>rpsM</i>	PCR, sequencing	RpsM_F	TTTGGGATGGCTGGATTTG
	PCR, sequencing	RpsM_R	GGGAAAAGCATTGAACACCA
<i>bla_{TEM1}</i>	PCR, sequencing	TEM1_F	GAAAATGAGACGTTGATCGG
	PCR, sequencing	TEM1_R	GCTTATTATCACTTATTCAG
<i>envZ</i>	PCR, sequencing	EnvZ_F	CTGGCGAAACTGTTTCATTG
	PCR, sequencing	EnvZ_R	CCGTATGGTGGGAAGAAGATC
<i>nmpC</i>	PCR, sequencing	nmpC_F	GAACTTATGCCACTCCGTCAT
	PCR, sequencing	nmpC_R	CGTCTGCACGGCATACTCCT
<i>cpxA</i>	PCR, sequencing	Cpx_F1	CGCCGAGTGGATTCTGGT
	PCR, sequencing	Cpx_F2	CATCTGGCGTGAATCGAGCT
	PCR, sequencing	Cpx_R1	CCGGAACGCAAAGACGGT
	PCR, sequencing	Cpx_R2	GCTAATAACAGAATGACAAACTGGA
<i>gfp</i>	Real-Time PCR	RT_GFP_F	TCCGTTCAACTAGCAGACC
	Real-Time PCR	RT_GFP_R	GAAAGGGCAGATTGTGTCTG
<i>bla_{TEM1}</i>	Real-Time PCR	RT_BLA_F	TGAATGAAGCCATACCAAACG
	Real-Time PCR	RT_BLA_R	ATCCGCCTCCATCCAGTC
<i>recA</i>	Real-Time PCR	RecA_real_F	TACCGAACATCACGCCAATC
	Real-Time PCR	RecA_real_R	GTATGATGAGCCAGGCGATG
<i>ompC</i>	Real-Time PCR	OmpC_real_F	CCGTTTTTGGCCCTGATACTG
	Real-Time PCR	OmpC_real_R	CTTTATGCAGCAGCGTGGTA
<i>ompF</i>	Real-Time PCR	OmpF_real_F	GGTATTCCCCTGACCGAAA
	Real-Time PCR	OmpF_real_R	AACGACCGGCGATAGTAAAA
<i>nmpC</i>	Real-Time PCR	nmpC_real_F	CGGGTAAAAACGCTGAAGTC
	Real-Time PCR	nmpC_real_R	ATCTTCACCGAAGGTGGTCA
Construction of T-BAG			
Insertion of <i>rpsM</i> - <i>gfp</i> - <i>cam^r</i> fragment into the Tn10 transposon on F ⁺ ₁₂₈ by linear transformation	RGC_lin_F		AAAACCTATCTATTTTATTTATCTTTCAAGCTCAATAA
			AAAGCCGCGGTAGCCTGGGGTAATGACTCTCTAGC
	RGC_lin_R		CGAAAAACCTAAAAAGAGCTTGCCGATAAAAAAG
			GCCAATTTATTGCTATTGACGTCATTTCTGCCATTTCATCC
Swapping of the <i>cam^r</i> gene with the <i>bla_{TEM-1}</i> gene by linear transformation	TEM1_lin_F		CACTACCGGGCGTATTTTTTTGAGTTATCGAGATTT
	TEM1_lin_R		TCAGGAGCTAAGGAATGAGTATTCAACATTTCCGT
			TATTGAGGCGTAGCACCAGGCGTTTAAGGGCA
			CCAATAACTGCCTTAAAATTACCAATGCTTAATCAGTG

Identification of duplication junctions		
	Rep31up_F	TGCGGTGGATATTCTCGTTGA
	Rep23down_R	CCTCCGCCTGATGCTCAAT
	IS3_1up_F	TGAAGTCGGGCTGTTTGGTAA
	IS3_2down_R	GTCGATTTGCTCACGGTTGA
Identification of Tn10dcam insertion point		
	TR_Cm	GGTGCGTAACGGCAAAAGCAC
	TL_Cm	CCAGAGCCTGATAAAAACGGTTAC
	Universal_Cm	GACAAGATGTGTATCCACCTTAAC
	Arb1	GGCCACGCGTCGACTAGTACNNNNNNNNNGATAT
	Arb6	GGCCACGCGTCGACTAGTACNNNNNNNNNACGCC
	Arb2	GGCCACGCGTCGACTAGTAC