ampC \(\beta\)-lactamase hyperproduction in Escherichia coli: Natural ampicillin resistance generated by horizontal chromosomal DNA transfer from Shigella

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Communicated by Robert L. Sinsheimer, July 25, 1983

ABSTRACT Six ampicillin-resistant clinical isolates of Escherichia coli that hyperproduced the chromosomal ampC \(\beta\)-lactamase were studied. By DNA sequence analysis, we found that five of them were identical over an entire 449-base-pair sequence and carried a novel strong ampC promoter [Olsson, O., Bergström, S. & Normark, S. (1982) EMBO J. 1, 1411-1416]. Except for one base pair this sequence was identical to that of a low \(\beta\)-lactamase-producing clinical isolate of Shigella sonnei. Spontaneous one-step mutants of S. sonnei that overproduced the ampC \(\beta\)-lactamase by 45-fold were characterized and found to be mutated at the single base that distinguishes S. sonnei from the five E. coli hyperproducers. The most likely explanation for this result is that chromosomal DNA was transferred in vivo from Shigella to E. coli across the species barrier.

Escherichia coli contains a chromosomal gene, ampC, that codes for the production of a class C \(\beta\)-lactamase (1). By using ampC DNA as a probe in DNA-DNA hybridization experiments it has been possible to detect DNA homologous to this gene, not only in clinical isolates of E. coli but also in other enterobacterial species (2). An especially strong signal was obtained when hybridizing to DNA from Shigella sonnei and Shigella flexneri.

Expression from the E. coli K-12 ampC gene is very low, and ampC wild-type strains are resistant to ampicillin at about 1 \(\mu\)g/ml. The low production of ampC \(\beta\)-lactamase in E. coli K-12 is due to a relatively weak promoter and to the presence of a transcriptional attenuator in the ampC leader (3). Transcriptional termination from the ampC attenuator decreases with increasing growth rate, so that expression from ampC is under control of growth rate (4).

By selection for ampicillin resistance in batch cultures of E. coli K-12, different classes of ampC \(\beta\)-lactamase hyperproducing mutants have been isolated. Up-promoter mutants, in each case increasing the fitness to the consensus sequence of E. coli promoters (5, 6), occurred at an incidence of between \(10^{-10}\) and \(10^{-11}\) and increased ampC expression 7- to 21-fold (3, 7). Other one-step genetic events known to increase E. coli K-12 ampC expression are (i) integration of insertion sequence IS2(II) into the \(-10\) hexamer of the ampC promoter, which increased expression 20-fold (incidence \(10^{-10}\) to \(10^{-11}\)) (8); (ii) ampC attenuator mutations, which increased expression 4-fold during fast growth (incidence \(10^{-3}\) to \(10^{-4}\)) (3), and (iii) gene amplification, which may increase expression 2- to 20-fold (incidence \(10^{-3}\) to \(10^{-11}\)) (9).

It has not been possible to increase expression from the E. coli K-12 ampC gene more than 21-fold by a single genetic event. To achieve an even higher level of expression from the E. coli ampC gene, two or more mutations have to be combined.

In view of these findings in E. coli K-12, we found it intriguing to elucidate how naturally occurring ampC hyperproduction may have evolved. Earlier we characterized six clinical E. coli isolates that produced 24- to 45-fold-increased levels of the ampC enzyme (10). By determining the sequence of the ampC regulatory regions and the surrounding DNA sequences of these \(\beta\)-lactam-resistant E. coli isolates and comparing them to wild-type E. coli and S. sonnei, we conclude that in five of the six E. coli isolates \(\beta\)-lactamase hyperproduction must have evolved by a single mutation from a Shigella ampC control region. This generates a novel, strong ampC promoter that differs from, but overlaps, the ampC promoter defined in E. coli K-12.

MATERIALS AND METHODS

Bacteria. The E. coli K-12 strain LA5 and the clinical isolates of E. coli and S. sonnei used in this study, the relative amounts of \(\beta\)-lactamase produced by these strains in rich media, and their LD\(_{50}\) value on rich ampicillin media are listed in Table 1. The E. coli strains C11, C13, C14, C15, C16, and C17 were obtained by screening 109 \(\beta\)-lactam resistant isolates from patients with urinary tract infections as previously described (10). E. coli strains C115 and C123 and S. sonnei strain OS10 were also isolated from clinical specimens and were considered as wild-type ampC strains on the basis of their low \(\beta\)-lactamase production. E. coli K-12 strain JM103 was used as a host for M13 phage growth (13).

Media. In most experiments bacteria were grown in minimal medium E (14) or in LB medium of Bertani (15). The carbon sources used are indicated in the legend to Fig. 1. Phage M13 was propagated on strain JM103 in YT medium (16).

DNA Techniques and Cloning of the ampC Gene. Isolation of chromosomal DNA was as described (2). Plasmid pBEU28 carrying kanamycin resistance and a temperature-sensitive replication control (17) was kindly provided by B.-E. Uhlin (this laboratory). Plasmid pNU78 has been described (2). Chromosomal DNAs from the E. coli strains C123, C115, C11, C13, C14, C15, and C17 and from the S. sonnei strains OS105, OS107, OS112, and OS113 (Table 1) were isolated, cleaved with EcoRI, ligated with EcoRI-digested plasmid pBEU28, and used to transform the \(\beta\)-lactamase-negative strain SN03. Transformants carrying the ampC gene were selected on ampicillin plates. The ampC gene of strain C16 was inserted into plasmid pNU78 as described (18). Hybrid plasmids obtained were characterized by physical mapping.

The ampC region from S. sonnei strain OS10 was isolated from a \(\lambda\) phage library as described (19) and inserted into plasmid pNU78.

Abbreviation: bp, base pair(s).
Table 1. Bacterial strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relative β-lactamase production</th>
<th>Ampicillin resistance, μg/ml</th>
<th>Comments</th>
<th>Source or ref.</th>
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<tr>
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<tr>
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<td></td>
<td></td>
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<td>C11</td>
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</tr>
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</tr>
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<td></td>
<td></td>
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<tr>
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<td>1.5</td>
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<tr>
<td>OS113</td>
<td>37</td>
<td>80</td>
<td>Mutant from OS10</td>
<td>2</td>
</tr>
</tbody>
</table>

* Determined by rocket immunoelectrophoresis according to Laurell (12).
† Ampicillin concentration permitting 50% of single cells to form colonies.

Plasmids carrying the ampC gene on an EcoRI fragment (Fig. 2) were prepared as described (18). Restriction enzyme fragments were prepared from polyacrylamide gels (30), and a 931-base-pair (bp) Sal I/Xho I restriction fragment was inserted into the M13 cloning vector Gort I (21). Plaques of phage carrying the ampC gene were identified by plaque hybridization (22). The M13 vector mp8 carrying the E. coli K-12 Sal I/Xho I fragment of the ampC control region (Fig. 2) inserted in either orientation was labeled by synthesizing the M13 complementary strand from a probe primer in the presence of [α-32P]dCTP (23) and were used directly as probes for plaque hybridization. Phage DNA was prepared from positive plaques, and its sequence was determined by the dideoxy method (24). Both strands of the DNA were analyzed, using two oligodeoxynucleotides as strand-specific primers.

Other Methods. Ampicillin resistance was determined for single cells as described (10). To quantitate ampC-β-lactamase expression, the immunoelectrophoresis method of Laurell was used (12).

RESULTS

Eight clinical E. coli isolates were investigated. Two of the isolates, E. coli C123 and E. coli C115, show the same level of ampC β-lactamase as E. coli K-12, whereas the remaining six isolates all hyperproduced the enzyme (Table 1). Only one isolate, E. coli C13, showed no growth-rate-dependent regulation (Fig. 1).

DNA Sequence Analyses of Clinical E. coli Isolates with Wild-Type ampC β-Lactamase Expression. Because E. coli K-12 was isolated in the pre-antibiotic-therapy era, two recent clinical isolates of E. coli (C123 and C115), resistant to ampicillin at 1 μg/ml, were randomly chosen for this study. This was done to determine whether or not the ampC control region of E. coli K-12 could still be regarded as a prototype for E. coli. The sequences of 449-bp DNA segments, including the ampC control region, from both of these strains were determined. In Fig. 2 it can be seen that they resemble each other more than they resemble the corresponding sequence of E. coli K-12. Unlike

![Fig. 1. Regulation of the ampC β-lactamase enzyme as a function of growth rate. The relative amount of β-lactamase, determined by rocket immunoelectrophoresis, is plotted as a function of the first-order constant for growth (k), as calculated from the expression k = ln 2/mass doubling time in hr. The media used were medium E + acetate, medium E + glycerol, medium E + glucose, and medium E + glycerol + Cas-

amino acids. Data are for E. coli clinical isolates C11 (●), C13 (○), C14 (▲), C15 (△), C16 (●), and C17 (■).
of the \textit{E. coli} consensus promoter. A mutation at this position in \textit{E. coli} K-12 is known to increase transcription initiation by a factor of about 20 (7). It is therefore likely that \textit{ampC} hyperproduction in \textit{E. coli} C13 is a combined effect of these two discrepancies from the prototype \textit{ampC} promoter and attenuator.

DNA Sequence Analyses of the \textit{E. coli} \textit{ampC} \textit{B}-Lactamase Hyperproducers with a Retained Metabolic Control. The five independently isolated \textit{ampC} \textit{B}-lactamase hyperproducers (C11, C14, C15, C16, and C17) that had retained growth-rate-dependent control were found to be identical over the entire 449-bp DNA segment. This sequence differed by 9 bp from the co-
responding sequence of *E. coli* K-12. With the *E. coli* ampC
prototype transcriptional start base as a reference point, these
differences occurred at positions −88, −82, −42, −18, −1,
+58, +65, +185, and +317 (Fig. 2). When aligned with C115
and C123, 13 and 12 differences were found, respectively (Fig.
2). Thus, the five identical β-lactamase hyperproducers (C11,
C14, C15, C16, and C17) had a sequence quite different from
the other *E. coli* sequences. We had previously demonstrated
that for *E. coli* C16, the T at position −42 and the A at −18
create a strong promoter distinct from, but overlapping, that
declared as the ampC prototype promoter (18). In *E. coli* C16,
transcription initiation starts four and five bases upstream from
the start in *E. coli* K-12 (18). Since *E. coli* C11, C14, C15,
and C17 are identical to *E. coli* C16 in the ampC region, in the level of β-lactamase production, and in the transcriptional initiation
site (data not shown), they must all use the strong ampC pro-
moter as defined by *E. coli* C16.

DNA Sequence Analyses of the ampC Region in *S. sonnei*.
Because DNA-DNA hybridization has not shown any detect-
able differences between the ampC regions from *E. coli* and *S.
sonnei* (2) and because their respective ampC gene products are
immunologically identical (2), the ampC region of the β-lactam-
sensitive clinical isolate *S. sonnei* OS10 was cloned (19). The
DNA sequence of the entire 449-bp segment defined in this
paper, carrying the ampC control region, was, apart for 1 bp,
identical to the sequence found in the five different *E. coli ampC*
hypercocius isolates (C11, C14, C15, C16, and C17) (Fig.
2). The difference was a C-G base pair in the OS10 sequence
instead of a T-A base pair, as found in the *E. coli* hyperpro-
curers, at position −42 relative to the *E. coli* K-12 transcrip-
tion start (Fig. 2). This difference is within the hexamer sequence
that we previously have defined as the −35 region of the
ampC promoter in *E. coli* C16 (18). This sequence reads 5'-C-T-G-A-
C-A-3' in *S. sonnei* OS10 and 5'-T-G-A-C-A-C-3' in the resis-
tant *E. coli* isolates.

DNA Sequence Analyses of Spontaneous *S. sonnei ampC*
β-Lactamase-Hyperproducing Mutants. *S. sonnei* OS10 is resis-
tant to ampicillin at 1 μg/ml and its production of *ampC* β-lac-
tamase is comparable to that of *E. coli* K-12 wild type (strain
LA5) and the *E. coli* clinical isolates C123 and C115. As in these
*E. coli* strains, the production of *ampC* β-lactamase of *S. sonnei*
OS10 increases with growth rate (2). Spontaneous ampicillin-
resistant mutants from *S. sonnei* OS10 were selected with ampi-
cillin at 5 μg/ml and were obtained at an incidence of 10−16.
Out of 20 mutants tested in rich medium, 18 were resistant to
ampicillin at 5–10 μg/ml and two were resistant to about 90
μg/ml. The ampC regions from the two representatives of the
more resistant group, *S. sonnei* OS112 and OS113, as well as
two mutants resistant to ampicillin at about 10 μg/ml, *S. sonnei*
OS106 and OS107, were cloned and their DNA sequences were
determined. The ampC regions of OS112 and OS113 were identical and differ from the parental strain OS10 only by a
C-G to T-A transition at position 142 relative to the *E. coli ampC*
prototype transcription start base (Fig. 2). Consequently, the
ampC regions of *S. sonnei* OS112 and OS113 are identical to
those found in the *E. coli* clinical isolates C11, C14, C15, C16,
and C17. In *vitro* transcription confirmed that these two ampi-
cillin-resistant *S. sonnei* mutants initiated transcription four
and five bases upstream from the *E. coli* K-12 initiation site (data
not shown), as earlier shown for *E. coli* C16 (18). Thus *S. sonnei*
OS112 and OS113 utilize the ampC promoter defined in *E. coli*
C16 (18). However, the parental *S. sonnei* OS10 uses the same
transcription initiation site as *E. coli* K-12 and the two clinical
*E. coli* ampC prototypes, since in *vitro* transcription gives the
41-nucleotide attenuated leader transcript (data not shown). Thus,
the novel strong promoter is generated solely by the base sub-
stitution at −42.

In *S. sonnei* OS107 a C-G to T-A transition at position −11
had changed the −10 region in the *ampC* prototype promoter
from 5'-T-A-C-A-A-3' to 5'-T-A-T-A-A-3' (Fig. 2). An identical base pair substitution in *E. coli* K-12 is known to in-
crease β-lactamase production about 7-fold (7). Finally, *S.
sonnei* OS106 was mutated in the ampC attenuator in that it carried
an insertion of a G-C base pair between positions +18 and +19
(Fig. 2). This alteration will decrease the thermodynamic sta-
ility of the hairpin structure by changing the free energy of forma-
tion from −29.4 kcal/mol to −21.4 kcal/mol (25). This is in agreement with the finding that this isolate has lost its growth
rate control of β-lactamase expression (2).

DISCUSSION

The purpose of this study was to deduce how increased expres-
sion from the *E. coli* chromosomal β-lactamase gene, *ampC*, can
evolve under natural conditions. We therefore characterized six
clinical isolates of *E. coli*. These isolates produced very high
levels of *ampC* β-lactamase, higher than we have been able to
select for as a single mutational event in *E. coli* K-12 under lab-
oratory conditions.

Here we demonstrate that five of these six clinical *E. coli*
isolates have obtained their *ampC* β-lactamase-hyperproducing
ability as a consequence of a natural gene exchange with *Shi-
gella*. Although independently isolated, they are all identical in
the 449-bp DNA sequence investigated. They differ markedly
from the corresponding sequences of *E. coli* K-12 and the other
clinical *E. coli* isolates. Furthermore, the five isolates con-
tained, relative to the prototype ampC promoter, the two base
pair substitutions that generate the novel 45-fold stronger ampC
promoter (18). *S. sonnei* OS10, which we assume carries a *Shi-
gella* ampC prototype promoter, was identical in the ampC con-
trol region to these five clinical β-lactamase hyperproducers
except for position −42 (Fig. 2). The expression from the *S.
sonnei* OS10 ampC promoter is very low (Table 1), and in *vitro*
transcription revealed that the same start base is used in this
strain as in the *E. coli* K-12 prototype promoter (data not shown).
However, the *Shigella* promoter has an A at position −18.
Therefore, a single mutational event (C to T) at −42 is sufficient
to shift transcription initiation from the prototype promoter to
the novel strong one. This unique property distinguishes the
*Shigella* promoter from that of the *E. coli* prototype and gives
*Shigella* the potential to mutate to a very high ampicillin resis-
tance level.

It can be argued that the ampC control region of *E. coli* K-
12 is not typical of *E. coli* strains present today. The five *E. coli*
ampC hyperproducers could have evolved from a recent *E. coli*.
However, the sequenced DNA of hyperproducers differed
markedly from that of two recent initial *E. coli* isolates with low
β-lactamase expression (C115 and C123). These latter isolates
used the ampC prototype promoter and showed only one base
pair difference in the promoter region relative to *E. coli* K-12
(position −28).

It can also be argued that a transfer has occurred to *S. sonnei*
of the high ampicillin resistance sequence present in *E. coli* C11,
C14, C15, C16, and C17. This sequence could subsequently
have mutated and generated the *S. sonnei* OS10 sequence, which
then would be an atypical *Shigella* sequence. We cannot ex-
clude this possibility, since it should require DNA sequence
analysis of a large number of *E. coli* and *Shigella* isolates. How-
ever, despite attempts with many different isolates, we have
never been able to isolate one-step mutants in *E. coli* with more
than a 21-fold increased ampicillin-resistance level. This suggests that E. coli isolates normally lack the A residue at position -18 that is required to generate the novel strong ampC promoter. On the other hand, different Shigella isolates are easily mutated to high ampicillin resistance, favoring the idea that the Shigella OS10 ampC promoter sequence represents the prototype for Shigella.

The sixth isolate, E. coli C13, however, most likely owes its 25-fold increased ampC production to one promoter and one attenuator mutation in an E. coli prototype sequence. E. coli C13 is also mutated in the ampC signal peptide at position +81, which may negatively affect the secretion or stability of the β-lactamase enzyme.

The ampC promoter overlaps the distal gene frdD, in the frd operon (26). The protein encoded by frdD is thought to act as a membrane anchoring protein for the fumarate reductase complex (27). It is possible that evolution of ampC hyperproduction can only proceed in such a way that it will not adversely affect the function of the frdD gene product. The -42 mutation in the Shigella sequence, generating the strong novel ampC promoter, will not affect the frdD gene product, whereas all known up-promoter mutations in the E. coli ampC prototype promoter will do so.

In our hands transfer of ampC-mediated ampicillin resistance between S. sonnei and clinical isolates of E. coli could easily be achieved in batch cultures either by phage P1 transduction or R-factor-mediated mobilization (data not shown). We favor the idea that a similar transfer has occurred in areas where shigellosis is endemic. We believe that such transfer has generated a small E. coli subpopulation containing the Shigella ampC prototype region. Strains carrying the novel ampC promoter are then selected from this subpopulation in the intestine by a small mutational event. Alternatively, β-lactamase resistance may have evolved already in Shigella and then transferred to E. coli.

In this study we have only compared 449-bp DNA segments. Therefore we do not now the sizes of the Shigella sequences that are present in the resistant E. coli isolates. If the Shigella E. coli hybrids have been generated by conjugation, these isolates may represent very large natural intergeneric hybrids. It is conceivable that such hybrids are quite common in nature, putting into question the traditional classification system of E. coli and Shigella.

In conclusion, naturally occurring chromosomal β-lactam resistance in E. coli may have evolved by horizontal transfer of ampC DNA from Shigella species. A single base pair substitution in the Shigella prototype ampC sequence, while not affecting the protein of the overlapping frd gene, appears to give rise to a level of resistance impossible to achieve by the E. coli prototype ampC sequence through a single mutational event.

We thank Stina Olofsson for excellent technical assistance. We are indebted to Richard Goldstein (Harvard) for valuable discussions and to Christopher Korch for carefully reading the manuscript. This work was supported by grants from the Swedish Natural Science Research Council (Dnr 3730), the Swedish Medical Research Council (Dnr 5428), and the Board for Technological Development (Dnr 81-5394).