Synthesis of Penicillin-Binding Protein 6 by Stationary-Phase Escherichia coli

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The level of penicillin-binding protein 6, a d-alanine carboxypeptidase I, was found to be 2- to 10-fold higher in stationary-phase cells than in exponentially growing cells of Escherichia coli. This increase appeared to be due to de novo synthesis rather than to an unmasking of preexisting material. There was no comparable change in the amount of any of the other six penicillin-binding proteins.

There has been significant progress in identifying probable roles for some of the seven major penicillin-binding proteins (PBPs) in the Escherichia coli cell membrane. Both biochemical and genetic data suggest that some of them are enzymes that are active in synthesis or modification of the peptidoglycan layer of the cell wall during cell elongation, shape maintenance, or septum formation (13, 17-19, 23-25). In an effort to further understand the in vivo roles and regulation of these proteins, we examined the PBPs from E. coli cells that had been incubated in a variety of growth conditions. Since a number of studies have already indicated the pleiotropic effects of growth medium and growth rate on cellular protein composition (1, 6, 10, 15, 22), some ambiguities and perhaps irrelevant fluctuations in the amounts of the individual PBPs may be observed with this approach. Therefore, we have confined this report to the one major change in a PBP which we found to be both unique and reproducible.

There is probably less known about the physiological role of PBP 6, the subject of this report, than about any of the other PBPs in E. coli. Analysis of the purified protein showed that it is primarily a d-alanine carboxypeptidase I, although its specific activity is substantially less than that of PBP 5 (2). Pratt et al. (21) have recently reported that PBP 6 is synthesized and processed in a manner which supports the notion that the protein functions on the outside of the membrane where the final steps in peptidoglycan synthesis occur. However, since no mutants lacking PBP 6 activity have been isolated, it remains uncertain whether it is actually required for normal cell wall synthesis. Here we report the fact that stationary-phase cells of E. coli continue to synthesize PBP 6 until it reaches a level two to ten times greater than the exponential-phase amount.

E. coli strains X975, obtained from R. Curtiss, and CP78, obtained from B. Bachmann, were used in this study. Their genotypes have been described previously (7, 12). Membranes were routinely prepared by differential centrifugation after sonic disruption of the cells (4, 5, 23). The PBPs were detected by fluorography of sodium dodecyl sulfate-polyacrylamide slab gels containing membrane proteins labeled with a saturating concentration of [14C]benzylpenicillin (Amersham Corp., Arlington Heights, Ill.; 2, 23, 25).

Initially, the PBPs of cells from two sets of conditions were compared: exponential phase with the stationary phase and minimal medium with the complex medium. Stationary-phase cells, in this instance, were harvested 12 to 18 h after exponential growth had ceased. The fluorograph in Fig. 1 is typical of the results obtained with E. coli X975. The data from several assays are presented in Table 1. Two findings are particularly noteworthy and were observed in both kinds of media. PBP 6 was always more abundant in stationary-phase cells than in exponential-phase cells, and PBP 3 appeared to be the most reduced of the PBPs in stationary-phase cells. An increase in the amount of PBP 6 during stationary phase seemed to be a general phenomenon, whereas the near total loss of PBP 3 was not always observed in other strains of E. coli grown under similar conditions (data not shown). Depending on the specific strain that was examined, the amount of PBP 6 per milligram of membrane protein in stationary-phase cells was 2- to 10-fold greater than the amount present in exponential phase. The growth medium did have some effect on the level ultimately reached by PBP 6 in stationary-phase cells. For example, there was always less of an increase in cells from minimal medium compared with cells from a complex medium (Table 1).

The increase in PBP 6 normally began within the first 2 h after exponential growth had ceased
FIG. 1. PBPs 1A to 6 of *E. coli* X975 analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and fluorography. Approximately the same amount of protein was added to each slot. Lane 1, PBPs from midexponential-phase cells grown at 37°C in a supplemented minimal salts medium (5) containing 0.4% glucose; lane 2, PBPs from late stationary-phase cells in the same minimal medium; lane 3, PBPs from midexponential-phase cells grown in Penassay broth (Difco Laboratories); lane 4, PBPs from late stationary-phase cells in Penassay broth. The fluorograph in the lower section is from a much shorter time of exposure of the same gel to a piece of X-ray film.

TABLE 1. Comparison of the amount of each PBP in membranes from cells harvested in stationary phase with the amount from cells harvested in exponential phase in two kinds of media

<table>
<thead>
<tr>
<th>PBP</th>
<th>Stationary phase/exponential phase ratio in:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Complex medium</td>
</tr>
<tr>
<td>1A</td>
<td>0.45</td>
</tr>
<tr>
<td>1B</td>
<td>1.08</td>
</tr>
<tr>
<td>2</td>
<td>0.43</td>
</tr>
<tr>
<td>3</td>
<td>0.13</td>
</tr>
<tr>
<td>4</td>
<td>0.96</td>
</tr>
<tr>
<td>5</td>
<td>0.85</td>
</tr>
<tr>
<td>6</td>
<td>3.90</td>
</tr>
</tbody>
</table>

Three separate cultures of cells were grown and harvested under each of the four conditions described in the legend to Fig. 1, and their membrane-bound PBPs were assayed. The PBPs were quantitated by scanning the fluorograph with a Biomed soft-laser scanning densitometer. Averages of the data from the three cultures in each set were used to calculate the values listed here.

When a culture that had been in stationary phase for 4 h was diluted into fresh prewarmed medium, exponential growth resumed with no detectable lag. PBP 6 rapidly declined to a level that was typical for the exponential-phase cells of that strain. When the cells re-entered stationary phase, the level of PBP 6 again increased (Table 3). It is not known whether the decline in PBP 6 was due to its extraction from the membrane or to its inactivation so that it could no longer be measured by the penicillin-binding assay.

We considered the possibility that PBP 6 was present in a constant amount throughout the

TABLE 2. Absolute amount of [14C]benzylpenicillin bound to PBPs 5 and 6 in membranes from cells harvested at different times

<table>
<thead>
<tr>
<th>PBP</th>
<th>Exp</th>
<th>0</th>
<th>2</th>
<th>4</th>
<th>6</th>
<th>8</th>
<th>21</th>
<th>C</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>7</td>
<td>8</td>
<td>7</td>
<td>6</td>
<td>6</td>
<td>7</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>6</td>
<td>8</td>
<td>10</td>
<td>43</td>
<td>44</td>
<td>53</td>
<td>57</td>
<td>52</td>
<td>4</td>
</tr>
</tbody>
</table>

The absolute amount of bound penicillin is expressed in arbitrary units of optical density which were determined by scanning a single fluorograph with a microdensitometer. PBPs 5 and 6 from cells of the *relA* E. coli strain CP78 grown in Penassay broth at 37°C and harvested during midexponential growth (Exp), at the time corresponding to cessation of exponential growth (0), and at various times (2, 4, 6, 8, and 21 h) thereafter. Part of the culture at time 0 was transferred to a separate flask and incubated with 200 μg of chloramphenicol per ml for 4 h (C). The optical density of the culture remained essentially unchanged during this time.
growth of the population but that it was partially
cryptic or "masked" during exponential phase.
This does not seem likely, however, since the
pattern of changes observed in membranes
prepared by sonication of cells (Fig. 1) was identical
to that seen in whole cells made permeable by
addition of treatment with ether (20, 26; data not shown).
In the latter preparation, cryptic cell wall enzymes
reportedly remain cryptic, whereas in the soni-
cated samples normally inactive enzymes are
sometimes unmasked (9, 20). Furthermore, addi-
tion of the protein synthesis inhibitor chloram-
phenicol to the culture at the beginning of sta-
tionary phase completely prevented the increase
in PBP 6 (Table 2). After 4 h of incubation in
the presence of chloramphenicol, there was always
less PBP 6 per milligram of membrane protein
than before the addition of the antibiotic. This
suggested that de novo synthesis of PBP 6 was
probably occurring in the untreated stationary-
phase cells.

It has been reported that peptidoglycan syn-
esthesis in E. coli is under stringent control
and therefore does not occur in amino acid-deprived
relA* strains (12). The decrease in peptidogly-
can synthesis has been correlated with reduced
levels of carboxypeptidase activity in the non-
growing cells (9, 11). Although PBPs 4, 5, and 6
all have carboxypeptidase activity (2, 13, 18), it
has not yet been determined which of them, if
any, is the stringently regulated one. To the
extent that stationary-phase cells can be said to
resemble amino acid-starved cells (8), the results
reported here suggest that PBP 6 could not be
the relevant enzyme since it increased during
stationary phase. An examination of the PBPs in
amino acid-starved cells is in progress to address
this question more directly.

There are estimated to be at least 600 mole-
cules of PBP 6 in the inner membrane of a
growing E. coli cell (23). The discovery that
stationary-phase cells can have up to 10 times
this level could provide a clue to the protein's
function, or at least to its importance within the

cell. Since synthetic events in stationary-phase
cells occur at the expense of less essential cell
components, which are degraded to provide the
necessary precursors (8, 16), the cost to the cell
for the synthesis of PBP 6 must be considerable.
Although this could imply that the protein is
especially needed by the cell at this time, it
could also reflect the nonspecific loss of some
control mechanism in the aging cells. In any
case, these unusually high levels of PBP 6 seem
to be incompatible with normal exponential
growth since the amount of PBP 6 dropped
rapidly as stationary-phase cells resumed
growth in fresh medium. In the only other situa-
tion where an elevated level of PBP 6 has been
described for E. coli, there was a corresponding
abnormal cellular morphology (14). However,
that observation was made in growing cells that
also had an abnormal unsaturated fatty acid
composition. In the studies reported here, where
the cells were in their normal stationary phase,
the increase in PBP 6 was not accompanied by
any shape changes.

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TABLE 3. Absolute amount of [14C]benzylpenicillin
bound to PBPs 5 and 6 after shift to fresh medium

| PBP  | Amt of [14C]benzylpenicillin bound at (h after
shift to fresh medium): |
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.5</td>
</tr>
<tr>
<td>5</td>
<td>3</td>
</tr>
<tr>
<td>6</td>
<td>16</td>
</tr>
</tbody>
</table>

* Samples were harvested at various times after dilution of a 4-h-old stationary-phase culture into fresh broth. Exponential growth resumed immediately and
continued for approximately 2 h. Thus, the 3- and 4-h samples are again from stationary-phase cells.


