Directed Evolution of Biosynthetic Pathways

RECRUITMENT OF CYSTEINE THIOETHERS FOR CONSTRUCTING THE CELL WALL OF ESCHERICHIA COLI

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We report that expansion of thioether biosynthesis in Escherichia coli generates sulfur-containing amino acids that can replace meso-diaminopimelate, the essential amino acid used for cross-linking the cell wall. This was accomplished by jointly overexpressing the metB gene coding for L-cystathionine γ-synthase and disrupting the metC gene, whose product, L-cystathionine β-lyase, is responsible for the destruction of L-cystathionine and other L-cysteine thioethers. As a result, meso-lanthionine and l-allo-cystathionine were produced endogenously and incorporated in the peptidoglycan, thereby enabling E. coli strains auxotrophic for diaminopimelate to grow in its absence. Thus, current techniques of metabolic engineering can be applied to evolving the chemical constitution of living cells beyond its present state.

The enzymes of biosynthetic pathways are not endowed with an absolute specificity. One can therefore consider that metabolism encompasses not only the proper enzyme substrates and products but also the side products resulting from enzyme action on homologs or analogs of substrates (1, 2). Such side products are probably tolerated by cells in trace quantities only, since they at best divert a fraction of metabolic flows, and at worst impair the structure and function of macromolecules by entering nucleic acid (3) or protein (4) synthesis. If, however, a latent metabolite could under certain circumstances fulfill an essential need in cell chemistry, the way would be open for establishing a biosynthetic pathway de novo (5). Both parts of this scenario have been accomplished independently. On one hand, abnormal metabolites have been generated by blocking (6), deregulating (4), or recombining (7) biosynthetic pathways. On the other hand, a few unnatural compounds have been shown to serve as growth factors for auxotrophic strains in place of a required metabolite (Ref. 8 and review therein). Bridging the gap between the two parts, we have constructed strains of Escherichia coli whose lack of meso-diaminopimelate in their cell wall is compensated by abnormal side products of the methionine biosynthesis pathway. This result demonstrates that current techniques of metabolic engineering (9, 10) can be applied to evolving the chemical constitution of living cells beyond its present state.

Sulfur links are conspicuously rare in the construction of biological polymers. The high reactivity of thiols and of sulfonium ions (11) in an aqueous medium such as the cytoplasm might be invoked to explain this fact. Thioethers, however, can be formed readily and remain stable in water (11). In addition, the sulfur atom of thioethers (-S-) makes versatile connections within organic molecules, with geometric parameters close to those of the methylene group (-CH2-) (12). Potent antagonists of various amino acids (e.g. norleucine, thialysine, thiasoleucine) have been prepared as analogs of their natural counterparts (methionine, lysine, isoleucine) taking advantage of this resemblance (12). Yet, with the notable exceptions of the coenzyme biotin and of the methionine side chain, thioethers seem to occur in bacteria only under the form of l-cystathionine, a precursor of methionine, or of derivatives of S-adenosylmethionine, and of rare posttranscriptional and post-translational modifications (11). Whatever evolutionary circumstances led to undersizing sulfur links (13), their very scarcity provides a potential for enlarging the set of chemical components in living cells.

The bacterial cell wall seemed propitious for attempting such experiments of molecular evolution. It is composed mainly of a peptidoglycan, i.e. a giant macromolecule of periodic structure whose basic unit, a disaccharide-pentapeptide, is polymerized linearly via the disaccharide motif and cross-linked laterally via the peptide motif (for review see Ref. 14). Any alteration of the basic unit results in a global change of peptidoglycan structure and properties, a condition that cannot be easily investigated in vivo with protein or nucleic acid macromolecules. Such global alterations of peptidoglycan structure are encountered in nature as conserved variations along phyletic lines (15) or acquired as mechanisms of resistance to cell wall-targeted antibiotics (16-18). In E. coli and most bacteria, the function of peptidoglycan cross-linking is fulfilled by meso-diaminopimelate (see Fig. 1). It is a vital function as cell confinement, shape, and division depend upon peptidoglycan being a net and therefore cross-linked (14). The overall synthesis of diaminopimelate requires six steps (for review see Ref. 19), the last one being the conversion by a specific epimerase of L-diaminopimelate into meso-diaminopimelate (Fig. 2a). The latter is then either incorporated to form the peptidoglycan precursor UDP-N-acetylmuramyl-tripeptide (20) or irreversibly decarboxylated into L-lysine (19). Bacteria have no alternative way to synthesize this protein amino acid. Growth of E. coli mutants

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bear a deletion of the dianiminopimelate pathway in the presence of lysine and in the absence of dianiminopimelate therefore provided an inescapable selection screen for the endogenous production of dianiminopimelate substitutes.

Our initial goal was to install a biosynthetic pathway for the thioether analog of dianiso-dianiminopimelate, dianiso-lanthionine (Fig. 1), in the metabolism of E. coli through the smallest possible number of genetic changes. We found that amplifying cysteine thioether formation in dianiminopimelate-less strains indeed caused the incorporation in the cell wall of dianiso-lanthionine and also of L-cystathionine and of its stereoisomer L-allo-cystathionine (see Figs. 1 and 2b).

MATERIALS AND METHODS

Growth of Bacterial Strains—The strains and plasmids used for this study are listed in Table I. Bacteria were routinely grown in MS minimal medium, a buffer solution, pH 7.3, containing 50 mM dipotassium phosphate, 20 mM ammonium chloride, 4 mM citric acid, 1 mM magnesium sulfate, 3 μM ferric chloride, 1 μM manganese chloride, and 1 μM calcium chloride. To this medium was added 2 g/liter glucose and when necessary amino acids at a final concentration of 1 mM. Growth media were solidified using 15 g/liter agar (Difco) for the preparation of plates. Liquid and solid cultures were incubated at 37 °C. Rich medium LB was prepared according to Miller (21).

Construction of metB-carrying Plasmids—The plasmid pMB41 was constructed by ligating the 1.3-kilobase NsiI-PvuII fragment containing the wild type metB gene from pMAD4 (22) between the PstI and HinclII sites of pUC9 (23). Ligation of the EcoRI fragment containing the spectinomycin-streptomycin resistance gene and religation afforded the plasmid pDA411. From the EcoRI fragment of pMB41, yielding plasmid pMB42. Construction of plasmids and transformation of bacteria were accomplished following standard protocols (25).

Construction of Strains with Defined Insertions in Chromosomal Genes—Allelic exchanges of chromosomal wild type genes with disrupted plasmid-borne genes using Hfr strains (26) and rec strains (27) were performed according to published procedures. The mutations were then dispatched by P1 transduction (21).

A Δmeta::cat allele was introduced into the E. coli chromosome as follows. The plasmid pMA61 was constructed by ligating a BamHI-BglII fragment from pMA6 carrying the meta gene (28) into pUC13. The cat cartridge from pSKS114, excised by HinclII, was then inserted into pMA61 within the reading frame of the meta gene (29), by excision of the 324-base pair MulI-ClaI fragment, filling with Klenow DNA polymerase (Boehringer Mannheim) and blunt end ligation, yielding pMA63. This latter plasmid was then transferred to the Hfr P801 strain BW6165 auxotrophic for arginine (30), and a resulting transformant was crossed with the F′ prototrophic strain MG1655, selecting for chloramphenicol resistance in minimal medium containing methionine. The Δmeta::cat strain β176 was thus obtained as an ampicillin-sensitive methionine-auxotrophic exconjugant.

A Δdapa::cat allele was constructed as follows. The 1.857-base pair NsiI-PstI fragment from pDA1 (31) was ligated into the PstI site of pUC9. Digestion by MluI and religation afforded the plasmid pDA41, bearing a 185-base pair deletion within the reading frame of the dapa gene. A SacI adapter was ligated into the unique MluI site, yielding pDA411. Then, the cat cartridge from pSKS114 (32) excised with SacI was ligated into the unique SacI site of pDA411. From the resulting plasmid, pDA413, the Δdapa::cat-containing fragment was excised by action of BamHI and HindIII and ligated into pNO1523 containing the wild type rpsL gene of E. coli, which confers dominant sensitivity to streptomycin (33). This yielded pDA415. Allelic exchange of the chromosomal wild type dapa gene for Δdapa::cat was performed by crossing the F′ rpsL strain pop4063 (see Ref. 8), which is resistant to streptomycin, with a pDA415 transformant of Hfr KL16 (see Ref. 8), selecting for chloramphenicol and streptomycin resistance in the presence of dianiminopimelate. The Δdapa::cat strain β224 was thus obtained as an ampicillin-sensitive dianiminopimelate-auxotrophic exconjugant.

A dapB::kan allele was constructed by introducing a kan cartridge from pUC4K (Pharmacia LKB Biotechnology Inc.) into the unique BglII site of pDB17 (34), yielding plasmid pDB172. This plasmid, linearized by EcoRI, was used for transforming the recB recC sbcB strain JC7623 (27), resulting in the dapB::kan strain β159.

A dapD::cat allele was constructed by introducing the cat cartridge from pSKS114 (32) into the unique PstI site of the dapD gene in pDD11, yielding pDD12. The plasmid pDD11 was constructed previously by inserting the 2.4-kilobase PvuII-EcoRI fragment from pDD1 (35) into pK19 (36) cut by HinclII and EcoRI. The plasmid pDD12, linearized by HindIII and BamHI, was used for transforming the recB recC sbcB strain JC7623 (27), yielding the dapD::cat strain β160.

Synthesis of Cysteine Thioethers—The pure stereoisomers L-lanthionine and dianiso-lanthionine were prepared by condensation of L-chloroalanine with L-cysteine and D-cysteine, respectively, according to a published procedure (37). L-allo-Cystathionine was obtained similarly by condensation of L-chloroalanine with D-homo cysteine thiolactone. L-Cysteine, D-cysteine, L-chloroalanine, D-homo cysteine thiolactone, L-diaminopimelate, L-lanthionine, L-cystathionine, meso-dianiminopimelate, meso-lanthionine, L-allo-cystathionine.

![Fig. 1. Structural relationships between stereoisomers of cysteine thioethers and of dianiminopimelate.](image-url)
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FIG. 2. Invasion of peptidoglycan by thioether metabolites. Panel a, aspartate-derived metabolism of wild type E. coli showing the biosynthesis pathways of diaminopimelate and lysine (leftmost reaction sequence) and of methionine (rightmost reaction sequence). Protein amino acids are underlined. Panel b, proposed pathways for lantionine and cystathionine incorporation into the peptidoglycan of met-suppressed dap strains. Dotted arrows indicate reaction steps of normal metabolism maintained in redesigned strains, and solid arrows show latent metabolic steps revealed by metB overexpression and metC inactivation. The exogenous supply of lysine is shown by an open arrow. Relevant genes are indicated. Question marks indicate metabolic pathways present in our strains whose number of steps and responsible genes are unknown (see "Results").

RESULTS

Conversion of L-Lanthionine into meso-Lanthionine by Diaminopimelate Epimerase—An exogenous supply of lantionine and lysine has been reported to satisfy the needs of diaminopimelate auxotrophs (41). Mixtures of the three L-, D-, and meso-stereoisomers of lantionine (Fig. 1) were apparently used in these studies. We first showed that dap strains can grow with lysine and pure L-lantionine. meso-Lanthionine was found exclusively in the hydrolysis products of the cell wall fractions from such cultures (see Fig. 4a). Derivative strains with an additional dapF mutation (38), thus defective in diaminopimelate epimerase, failed to grow with L-lanthionine (Table II). Consistently, in vitro production of meso-lanthionine from L-lanthionine could be demonstrated with extracts from a dapF strain but not from the corresponding dapF strain (Fig. 3). Having proven that peptidoglycan construction is possible by using derivatives of L-lanthionine through diaminopimelate epimerase (Fig. 2), we undertook to establish the synthesis of this compound in the metabolism of E. coli. Several enzymes able to effect the condensation of various nucleophilic groups on the C8 moiety of serine through formation of a pyridoxal phosphate adduct stood as potential start points for evolving a lantionine synthase. Thus, the trpB-encoded subunit of tryptophan synthase from E. coli has been reported to react in vitro with mercaptopropanol, to give hydroxyethylcysteine; cysK-encoded cysteine synthase

thiolactone hydrochloride, L-homocysteine hydrochloride, and L-cystathionine were purchased from Sigma.

Enzyme Assays—Epimerization of L-lanthionine was assayed as reported previously for L-diaminopimelate (38).

Analysis of Cell Walls—Purification of peptidoglycan and determination of its composition using an automatic amino acid analyzer were as described previously (20, 39). Lanthionine and cystathionine stereoisomers were separated and quantitated by a high performance liquid chromatography procedure using a reversed-phase LiChrosorb RP18 column after chiral derivatization as described (40) with Marfey's reagent (1-fluoro-2, 4-dinitrophenyl-5-L-alaminamide).
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TABLE I

| Plasmid   | Markers                      | Construction                     |
|-----------|------------------------------|----------------------------------|
| pMB41     | bla(> metB(> metB(>         | See "Materials and Methods"       |
| pMB42     | bla(> oadA(>                | See "Materials and Methods"       |
| pDA415    | bla(> dapA(>                | See "Materials and Methods"       |
| pDB172    | bla(> dppB(>                | See "Materials and Methods"       |
| pDD12     | kan(> dapD(>                | See "Materials and Methods"       |
| pMA63     | bla(> metA(>                | See "Materials and Methods"       |
| pUC9      | bla                          | Laboratory collection, see Ref. 23 |

| Strain     | Relevant genotype            | Construction                     |
|------------|------------------------------|----------------------------------|
| JC7623     | recB recC sbcB              | Laboratory collection, see Ref. 27 |
| JCdapF     | recB recC sbcB dapF::MudII(cat) | Laboratory collection, see Ref. 38 |
| MG1655     | F-                           | Gift of Dr. B. Bachmann          |
| NK6027     | metC162::Trn10(tet)         | Gift of Dr. B. Bachmann          |
| W3110      | F- IN(rrnD-rrnE)             | Allelic exchange with pDB172 in strain |
| 8159       | dapB::kan                    | JC7623                           |
| 8160       | dapD::cat                    | Allelic exchange with pDD12 in strain |
| 8161       | dapB::kan                    | Transduction of MG1655 with lysate from 8159 |
| 8165       | dapB::kan metC::tet          | Transduction of 8161 with lysate from 8160 |
| 8166       | dapB::kan metC::tet pUC9     | Transformation of pUC9 with pUC9 |
| 8167       | dapB::kan dapD::cat          | Transformation of pUC9 with pUC9 |
| 8169       | dapB::kan dapD::cat metC::tet| Transformation of pUC9 with pUC9 |
| 8176       | metA::cat                    | Allelic exchange with pMA63 in strain |
| 8180       | metA::cat                    | Transduction of MG1655 with lysate from 8176 |
| 8183       | dapB::kan dapF::cat          | Transduction of 8183 with lysate from 8180 |
| 8185       | dapB::kan dapF::cat metC::tet| Allelic exchange with pDA415 in strain |
| 8194       | dapB::cat                    | Transduction of W3110 with lysate from 8194 |
| 8203       | dapB::kan dapD::cat metB(>   | Transformation of pMB42 with pMB42 |
| 8204       | dapB::kan metC::tet pMB42 (metB(> | Transformation of pMB42 with pMB42 |
| 8205       | dapB::kan dapF::cat pMB42 (metB(> | Transformation of pMB42 with pMB42 |
| 8224       | dapA::cat                    | Allelic exchange with pDA415 in strain |
| 8243       | dapA::cat metC::tet          | Transformation of MG1655 with lysate from 8243 |
| 8244       | dapA::cat metC::tet pUC9     | Transformation of MG1655 with lysate from 8244 |
| 8250       | metA::cat metC::tet          | Transformation of MG1655 with lysate from 8250 |
| 8251       | metC::tet                    | Transformation of MG1655 with lysate from 8251 |
| 8252       | metA::cat metC::tet pMB42 (metB(> | Transformation of MG1655 with lysate from 8252 |
| 8253       | dapA::cat metC::tet pMB42 (metB(> | Transformation of MG1655 with lysate from 8253 |
| 8254       | metC::tet pMB42 (metB(>      | Transformation of MG1655 with lysate from 8254 |
| 8255       | metC::tet pUC9               | Transformation of MG1655 with lysate from 8255 |
| 8256       | dapB::kan pMB42 (metB(>      | Transformation of MG1655 with lysate from 8256 |

To react O-acetylserine with triazole, to give triazolylalanine; and cysM-encoded cysteine synthase to react O-acetylserine with thiosulfate to give sulfocysteine (for review see Ref. 42). We focused our attention on cystathionine synthase, a pyridoxal phosphate enzyme of the methionine pathway encoded by the metB gene (22) which condenses the Cq moiety of homoserine on the thiol of cysteine: L-cysteine + L-cysteine + 0-succinyl-L-homoserine → L-cystathionine + succinic acid.

Cystathionine synthase displays a broader range of activities (43), and the enzyme from Salmonella typhimurium has been reported to form traces of L-lanthionine in vitro from L-cysteine alone (44). We chose to amplify in vivo this marginal and presumptive reaction, L-cysteine + L-cysteine → L-lanthionine + H2S, and to establish it as a bona fide biosynthetic pathway.

Bypass of Cystathionine in Methionine Biosynthesis—It seemed unlikely that overproduction of cystathionine synthase from a multicopy plasmid bearing the metB gene would be sufficient to sustain L-lanthionine production for two reasons. First, cystathionase, the metC-encoded enzyme that normally cleaves L-cystathionine to L-homocysteine, the immediate precursor of methionine (45), has a high activity toward lanthionine (46). Second, diaminopimelate shortage is lethal to growing dap mutants because the expansion of the
peptidoglycan surface cannot match the volume increase in such strains, which results in the rupture of cells (47). Growth should therefore be limited by a factor other than lanthionine production. Introduction of a metC null mutation offered the double advantage of preventing degradation of endogenously synthesized lanthionine and of slowing growth in the absence of exogenous methionine. We found that growth of a strain carrying a disrupted metC gene was restored by overexpression of the metB gene from a plasmid (compare strains β251 and β254 in Table III). A possible explanation could reside in a marginal cystathionine γ-lyase activity of MetB as suggested by the resemblance of MetB and MetC sequences (45). However, we also found that the growth of a metC strain carrying a null mutation of the metA gene, thus blocking the supply of the MetB substrate O-succinylhomoserine, was not restored by the introduction of a plasmid overexpressing metB in the presence of exogenous L-cystathionine (strain β252 in Table III). Growth of the metA strain β180 with L-cystathionine (Table III) ensured that this compound could be taken in the presence of exogenous L-cystathionine (strain p253 carrying the pMB42 plasmid was found to be about 140 min, to be compared with 75 min for the control strain p244 carrying the pUC9 plasmid exogenously supplied with methionine, lysine, and diaminopimelate directly onto minimal glucose medium with lysine resulted in low recovery of colonies. Higher recovery was achieved by passage onto medium containing lysine and diaminopimelate or lanthionine but lacking methionine, prior to restreaking onto medium containing only lysine. Once acclimatized, bacteria of met-suppressed dap strains could be propagated indefinitely in glucose minimal medium plus lysine, as liquid or solid cultures. The division time of the dapA derivative (38) of strain JC7623 afforded no detectable amounts of meso-lanthionine after 24 h (not shown).

Bypass of Diaminopimelate in Peptidoglycan Biosynthesis—As shown in Table IV, the dapB metC strain β204, bearing a plasmid overexpressing metB (hereafter designated met-suppressed dap strains), grew in minimal glucose medium containing lysine but no diaminopimelate. All the alleles of dapA, dapB and dapD conferring diaminopimelate auxotrophy which we tested could be suppressed by overexpression of an active metB gene overexpressed from the plasmids pMB41 or pMB42 in a metC defective context (data not shown). No suppression could be observed when the control plasmid pUC9 or other plasmids lacking an active metB were used instead of pMB41 or pMB42 (see strains β165 and β166 in Table IV). Consistently, we observed that pMB42 persisted in serial subcultures of met-suppressed dap strains in the absence of the requirement for lysine (Table IV) and therefore harbored no auxiliary synthesis of diaminopimelate whatsoever. Transferring bacteria of such strains from a medium with diaminopimelate and methionine directly onto minimal glucose medium with lysine resulted in low recovery of colonies. Higher recovery was achieved by passage onto medium containing lysine and diaminopimelate or lanthionine but lacking methionine, prior to restreaking onto medium containing only lysine. Once acclimatized, bacteria of met-suppressed dap strains could be propagated indefinitely in glucose minimal medium plus lysine, as liquid or solid cultures. The division time of the dapA metC strain β253 carrying the pMB42 plasmid was found to be about 140 min, to be compared with 75 min for the control strain p244 carrying the pUC9 plasmid exogenously supplied with methionine, lysine, and diaminopimelate, and with 90 min for the same strain supplied with methionine, lysine, and L-lanthionine (data not shown). No obvious changes in cell morphology or antibiotic susceptibility could be observed by routine comparison between met-suppressed dap strains and the wild type. As expected, diaminopimelate auxotrophs overexpressing metB and bearing an active metC gene, thus capable of degrading cysteine thioethers, did not grow on medium containing only lysine (strain β256 in Table IV). The addition of exogenous methionine was found to cause the lysis of met-suppressed dap strains in medium containing only lysine (strain β204 in Table IV), in agreement with the  

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**Table II**

**Supplementation of diaminopimelate auxotrophs by lanthionine stereoisomers**

| Strain | Genotype | Amino acid added to growth medium |
|--------|----------|----------------------------------|
| β161   | dapB     | +                                 |
| β183   | dapB dapF| –                                 |

**Table III**

**Suppression of cystathionase deficiency by overproduction of cystathionine synthase**

| Strain | Genotype | Amino acid added to growth medium |
|--------|----------|----------------------------------|
| β254   | metC     | +                                 |
| β252   | metC metA| –                                 |
| β255   | metC pUC9| –                                 |
| β251   | metC     | –                                 |
| β180   | metA     | +                                 |

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**Fig. 3. Epimerization of lanthionine stereoisomers.** A crude enzymatic extract of strain JC7623 (27), bearing an active dapF gene, was incubated with L-lanthionine at 25 °C as reported previously for L-diaminopimelate (38). Samples taken at times 0 h (open circles), 7 h (solid squares), and 24 h (solid circles) are shown. Extracts from the dapF derivative (38) of strain JC7623 afforded no detectable amounts of meso-lanthionine after 24 h (not shown).
Hypothesis that the growth limitation set by the metC mutation was necessary for the viability of such strains. Substitution of diaminopimelate for lysine, thus restoring normal metabolism of peptidoglycan, alleviated methionine toxicity (Table IV). All of these genetic traits were consistent with the endogenous synthesis of lanthionine at a low rate. However, we expected a dapF mutation to cause the lysis of met-suppressed dap strains provided with only lysine by blocking the conversion of endogenous L-lanthionine into meso-lanthionine, as it did for diaminopimelate auxotrophs exogenously supplied with L-lanthionine (strain 82183 in Table II). Introduction of a disrupted dapF allele into the met-suppressed dapB strain 8204 afforded a strain, 8205, whose growth was severely but not completely impaired on lysine plates (Table IV). This suggested that the peptidoglycan of met-suppressed dap strains might contain cross-linking amino acids other than meso-lanthionine.

Inventory of Cysteine Thioethers Fixed in the Peptidoglycan—Chemical analysis demonstrated the presence of meso-lanthionine in the peptidoglycan from met-suppressed dap strains (Fig. 4b; strains 8203 and 8253 in Table V). However, the amount of this compound was apparently reduced by a factor of about 3 relative to cell wall fractions from the dapA metC strain 8244 supplemented with exogenous L-lanthionine (compare Fig. 4, a and b). Elution profiles of peptidoglycan hydrolysates from met-suppressed dap strains also revealed an additional peak (Fig. 4b), which was tentatively identified as cystathionine stereoisomers using standards. Formal identification and quantitation were accomplished by using a different elution system (data not shown) and by chiral derivatization of peptidoglycan hydrolysis products (Table V).

Both methods showed the presence of about a 0.2 muramic acid equivalent of L-cystathionine together with a 0.4 equivalent of L-allo-cystathionine. A fraction of about a 0.3 muramic acid equivalent of meso-lanthionine completed the inventory of diaminopimelate substitutes in the peptidoglycan of the two met-suppressed dap strains 8203 and 8253 (Table V). No trace of meso-diaminopimelate or L-diaminopimelate could be detected in any of the numerous dap strains that we analyzed, unless these strains were exogenously supplied with these compounds (Fig. 4c and Table V). Altogether, it could be concluded that three bioisotopic reactions were catalyzed by the total pool of cystathionine γ-synthase expressed from both the chromosomal and plasmidic metB genes in met-suppressed dap strains. These reactions included the production of L-homocysteine or a derivative of it as a precursor of L-methionine for protein synthesis, as well as the production of L-lanthionine and of L-allo-cystathionine, respectively, for peptidoglycan synthesis (see Fig. 2b). Like L-lanthionine and meso-lanthionine, L-allo-cystathionine (Fig. 1) had not been reported previously to occur in the metabolism of wild type
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TABLE V

Peptidoglycan composition of met-suppressed dap strains

| Strain and genotype | Diaminopimelate | L-Lanthionine | L-Cystathionine | L- allo-Cystathionine |
|---------------------|-----------------|---------------|-----------------|----------------------|
| DapB metC pMB42     | 1.0             | 1.0           | 1.0             | 1.0                  |
| DapA metC pUC9      | 1.0             | 1.0           | 1.0             | 1.0                  |
| DapA metC pUC9      | 1.0             | 1.0           | 1.0             | 1.0                  |
| DapA metC pUC9      | 1.0             | 1.0           | 1.0             | 1.0                  |
| DapA metC pUC9      | 1.0             | 1.0           | 1.0             | 1.0                  |

TABLE VI

Supplementation of diaminopimelate auxotroph by cystathionine stereoisomers

The minus sign indicates no visible growth after 48 h or material that failed to grow after restreaking; the plus signs indicate thriving colonies. Bacterial growth was assessed at 37 °C on plates of MS minimal medium containing 2 g/liter glucose, 1 mM L-lysine, 1 mM L-methionine, and supplemented with 1 mM amino acids as indicated. Omitting methionine had no effect on the growth of strain β161. The same growth responses as for β161 and β165 were obtained with the dapA strain β194 and the dapA metC strain β243, respectively (see Table I).

| Strain | Genotype | Amino acid added to growth medium |
|--------|----------|----------------------------------|
| β165   | dapB metC| L-Cystathionine L- allo-Cystathionine |
| β161   | dapB     | -                  |
| β185   | dapB metC| +                  |

or mutant E. coli strains, to the best of our knowledge.

Cystathionine as a Cell Wall Building Block—The detection of cystathionine stereoisomers in peptidoglycan hydrolysates from met-suppressed dap strains prompted us to investigate whether such compounds could be utilized in replacement of diaminopimelate, when supplied exogenously. A thorough survey of the early literature on cell wall metabolism revealed that cystathionine had been reported to have a protective effect against lysis of lysE strains, as was lanthionine (47). This observation was apparently not followed by investigation of cystathionine incorporation in the peptidoglycan. All of the diaminopimelate auxotrophic strains of E. coli which we tested could grow with L- allo-cystathionine in the presence of lysine (Table VI). L- allo-Cystathionine could be recovered as a 0.9 equivalent of muramic acid together with a 0.1 equivalent of the physiological stereoisomer L-cystathionine in the peptidoglycan of such a strain (β244 in Table V). Exogenously supplied L-cystathionine could also satisfy the growth requirement of dap mutants in the presence of lysine, provided that the cystathionase gene metC was defective (Table VI). The division time of the dapA metC strain β244 grown with L-cystathionine in the presence of lysine and methionine amounted to about 240 min. Peptidoglycan extracts from such a culture were found to contain a mixture of a 0.4 muramic acid equivalent of L-cystathionine and a 0.6 equivalent of L- allo-cystathionine (Table V). The lower amount of D+L-alanine which we observed in this case (Table V) is presumably caused by less efficient cross-linkage or by higher liability of peptidoglycan monomers to carboxypeptidases. Recovery of both L-cystathionine and L- allo-cystathionine in the peptidoglycan of strain β244 exogenously supplied with either stereoisomer (Table V) attests to the existence of a functional epimerization pathway for the C4 moiety of L-cystathionine in the metabolism of E. coli. Comparison of the growth patterns of strains β165 and β185 in Table VI shows that an active dapF-encoded diaminopimelate epimerase is not required for the utilization of exogenous L-cystathionine or L- allo-cystathionine for peptidoglycan biosynthesis, as opposed to the utilization of L-lanthionine (strain β183 in Table II). This is in line with the marginal growth on lysine-containing medium of strain 8205, of genotype dapB dapF metC pMB42 (metB'), which presumably contains L-cystathionine and L- allo-cystathionine but no meso-lanthionine in its cell wall. The fragility of this strain under such growth conditions (Table IV), however, prevented us from proving this important point.

DISCUSSION

Protocols of directed evolution and metabolic engineering have been reported for establishing the conversion of xenobiotics into essential metabolites and for restoring the biosynthesis of essential metabolites in microorganisms (for a review see Refs. 9 and 10). The next logical step in broadening cell chemistry is to evolve organisms constructed with novel metabolites, i.e. noncanonical building blocks which are both endogenously produced and indispensably utilized. Breeding chemically redesigned descendants of microorganisms offers the prospect of synthesizing novel types of biopolymers as well as that of establishing strains unable to sustain growth
in natural habitats or, conversely, able to colonize inhospitable environments. Living organisms with chemical traits departing from those conserved among extant species should also provide experimental models for investigating the evolutionary processes that brought about such conserved traits. In an attempt to test the feasibility of this approach, we could demonstrate that a few coarse genetic changes are sufficient to remodel the cell wall chemistry and thereby the macromolecular distribution of sulfur in a bacterium through the recruitment of abnormal thioether metabolites.

That potentially useful compounds such as meso-lanthionine and L-allo-cystathionine can be so expediently generated begs the question of why they are generally avoided in the construction of wild type bacteria. Restriction of cysteine thioether usage could be because of constraints of enzyme specificities. The active conversion of L-cystathionine into L-homocysteine by cystathionase, the metC gene product, prevents its incorporation into peptidoglycan precursors. Inadvertently synthesized L-cysteine thioethers such as L-allo-cystathionine, L-lanthionine, and meso-lanthionine are probably not utilized by the cell, and it is therefore possible that a mutC mutation enhances the growth of dap strains exogenously supplied with L-lanthionine, meso-lanthionine, or L-allo-cystathionine as the cross-linking amino acid and is absolutely required for growing such strains with exogenous L-cystathionine (Table VI). The broad activity of cystathionase, which is indeed known to degrade generically L-cysteine thioethers in vitro (46), can thus be rationalized as fulfilling a corrective task, which adds to the biosynthetic function of the enzyme in E. coli metabolism (19). In addition, penicillin-binding proteins display a high specificity toward the D-amino acid center of meso-diaminopimelate for effecting cross-links in the peptidoglycan (49). This may explain why we isolated no strain whose peptidoglycan contains mainly the physiological L-stereoisomer of cystathionase (Table V). It has been found previously that the fraction of L-diaminopimelate incorporated in the peptidoglycan of dapF mutants, impaired in diaminopimelate epimerization, was excluded from cross-links (40). Low activity of the murE-encoded diaminopimelate-adding enzyme or of any enzyme acting at a later stage of cell wall synthesis (20) on L-cystathionine or its derivatives may also limit incorporation of this physiological stereoisomer in the peptidoglycan of dap mutants.

Restriction of cysteine thioether usage could also be caused by constraints of peptidoglycan structure. There are four main stereochemical contexts in which meso-diaminopimelate is embedded in the cell wall of wild type E. coli: (i) as the amino moiety of all cross-links; (ii) as the acyl moiety of certain cross-links; (iii) as laterally unconnected; (iv) as linker to lipoprotein (14). It is likely that formation of the septum and of caps involves further contexts (50). All of these stereochemical contexts are created and undone by at least seven enzymes of the periplasm (50), to be added to the four enzymes of the cytoplasm and cytoplasmic membrane (20) forming the successive covalent derivatives of meso-diaminopimelate prior to its insertion into the peptidoglycan. meso-Lanthionine has been found to replace partially or completely meso-diaminopimelate in the cell wall of certain species of Fusobacterium (51). Thorough studies with cells of Fusobacterium nucleatum have also shown that meso-lanthionine is not constructed in situ but incorporated as a free metabolite into peptidoglycan precursors (52), just as meso-diaminopimelate in wild type E. coli. These facts can be taken altogether as a natural demonstration of the stereochemical compatibility between the two compounds. The recruitment of meso-lanthionine in the peptidoglycan of E. coli corroborates this view. The fact that E. coli strains containing cystathionine stereoisomers in their peptidoglycan remain viable cannot be as easily reconciled with current views of peptidoglycan biochemistry and evolution. Cystathionine is one link longer than diaminopimelate and lanthionine (Fig. 1), which should distort stereochemical contexts throughout peptidoglycan structure and biosynthesis. We expected that even more chaotic configurations should result from the coexistence of meso-lanthionine, L-allo-cystathionine, and L-cystathionine in the peptidoglycan net. That no cystathionine stereoisomer had ever been observed in the cell wall of any species further suggested that progressive transition to cystathionine-based peptidoglycan was barred by natural selection. Quite surprisingly, this barrier could be overcome at a small genetic cost.

The genetic changes that we enforced in our strains for remodeling their chemical constitution implied no alteration of enzyme specificities or catalytic activities. Only the disruption of two genes (metC and either dapA, dapB, or dapD) and the overexpression of others (metE) were required. It should be noted that those remodeled strains whose dap genes have been deleted are unlikely to return to the chemical constitution of E. coli as long as they will be propagated in media lacking diaminopimelate. These strains can thus be viewed as having undergone an evolutionary commitment to use cysteine thioethers for building their cell wall. Although this commitment did not result from natural selection but was rationally set up in their genome, the fitness of the committed strains might now be improved by natural selection, for instance in long term chemostats. Rationally specializing cysteine thioether pathways (Fig. 2B) for producing exclusively meso-lanthionine or L-allo-cystathionine also seems worth attempting to study further the metabolic and macromolecular designs that were not explored, or not retained, during evolution.

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