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Molecular Cloning, Characterization, and Chromosomal Localization of *dapF*, the *Escherichia coli* Gene for Diaminopimelate Epimerase

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The *Escherichia coli dapF* gene was isolated from a cosmid library as a result of screening for clones overproducing diaminopimelate epimerase. Insertional mutagenesis was performed on the cloned *dapF* gene with a mini-Mu transposon, leading to chloramphenicol resistance. One of these insertions was transferred onto the chromosome by a double-recombination event, allowing us to obtain a *dapF* mutant. This mutant accumulated large amounts of LL-diaminopimelate, confirming the blockage in the step catalyzed by the *dapF* product, but did not require *meso*-diaminopimelate for growth. The *dapF* gene was localized in the 85-min region of the *E. coli* chromosome between *cya* and *uvrD*.

meso-Diaminopimelate (*meso*-DAP) is the direct precursor of lysine and is an essential component of the cell wall peptidoglycan in gram-negative bacteria. The formation of this key intermediate is catalyzed by DAP epimerase, an enzyme found several years ago in *Escherichia coli* (2) but only recently purified and studied for its catalytic properties (34). Furthermore, this enzyme has been specifically studied as a target for antibacterial effects (15).

As a step toward an understanding of the regulatory pattern of the whole lysine-DAP biosynthesis pathway in *E. coli*, we have begun to study the structure and expression of the gene for DAP epimerase (*dapF*, following the nomenclature of Bukhari and Taylor [6]). No mutants blocked in this enzymic step are available for gene cloning experiments. Consequently, we followed the method of Mechulam et al. (22), screening an *E. coli* cosmid library and assuming that a strain harboring a multicopy plasmid carrying the *dapF* gene would overproduce DAP epimerase. The gene was thus cloned and further localized by insertional mutagenesis. A mutation in the cloned gene allowed us to obtain for the first time a chromosomal *dapF* mutant. This mutation was then used for genetic mapping of the *dapF* locus on the *E. coli* chromosome.

MATERIALS AND METHODS

E. coli strains and plasmids used in this study are listed in Table 1. General genetic and cloning techniques have been described previously (11, 25).

DAP epimerase assay. DAP epimerase was assayed by the epimerase-catalyzed release of ^3H to water from [G-^3H]DAP after a 40-min incubation at 25°C as described previously (34). Typically, 100 μl of a reaction mixture containing 0.1 M Tris hydrochloride (pH 7.8), 1 mM EDTA, 1 mM dithiothreitol, and 0.5 μCi of (DL plus *meso*)-2,6-diamino[G-^3H]pimelic acid dihydrochloride (Radiochemical Centre, Amersham, U.K.) (1 Ci/mmol) was acidified with 500 μl of 10% trichloroacetic acid and applied to a column (1 ml) of Bio-Rad AG50W-X 4 ion-exchange resin (H^+ form). The column was washed three times with 500 μl of water, and the eluates were combined and counted for radioactivity. Protein was measured by the Bradford procedure (5). Specific activities are expressed as counts per minute of ^3H_2O liberated from DAP per milligram of protein per minute.

Screening of the cosmid library. A total of 450 clones from the *E. coli* genomic library constructed by Mechulam et al. (22) in the pHC79 cosmid were screened for DAP epimerase activity in the following way. Cultures (1 ml) grown in LB medium (25) in the presence of ampicillin (50 μg/ml) were harvested in late-log phase and centrifuged, and the pellets were stored at −20°C. Crude extracts were obtained by sonic disruption of the pellets suspended in 20 μl of buffer (20 mM Tris hydrochloride [pH 7.0], 1 mM EDTA, 1 mM dithiothreitol) followed by centrifugation (2 min in an Eppendorf microfuge). Protein concentrations were determined by using the Bradford procedure (5) on these supernatants, and groups of five extracts were pooled on the basis of approximately equivalent protein concentration values. DAP epimerase was assayed from all groups, and mean specific activity was determined. Two groups of five clones showed an overproduction of more than 25%, and each clone was then assayed individually.

Insertional mutagenesis. The in vivo method developed by Castilho et al. (7) with deleted derivatives of the Mu bacteriophage was used to mutagenize the cloned *dapF* gene. The Mu *d*II PR13 transposon has been constructed by P. Ratet (Thèse 3e cycle, Université Paris-Sud, Orsay, 1985). This mini-Mu can transpose only if complemented by the wild-type Mu bacteriophage. It carries the *cat* gene from Tn*9*, which confers resistance to chloramphenicol, and the lactose operon lacking the first eight codons of the *lacZ* gene and its upstream regulatory elements. This transposon can create gene fusions between the control elements of the gene into which insertion occurs and the coding region of the β-galactosidase gene conferring a Lac^+ phenotype.

Plasmid pDF3 was introduced into strain JM108(Mu *c*ts) harboring a chromosomal copy of Mu *d*II PR13. A mixed-phage stock was produced by thermoinduction of the Mu *c*ts and used in a plasmid transduction experiment as described by Castilho et al. (7). Insertions of Mu *d*II PR13 in pDF3 were selected in strain M8820(Mu) on LB plates containing ampicillin (50 μg/ml), chloramphenicol (25 μg/ml), and 5-bromo-4-chloro-3-indolyl-β-D-galactoside (40 μg/ml).

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Genotype	Source or reference
E. coli K-12		
IBPC111	F⁻ Δ(*pro-lac*) *argE metB ara gyrA rpoB supE recA1*	22
JM108(Mu cts)	Δ(*pro-lac*) *thiA gyrA96 endA1 hsdR17 relA1 supE44 recA1* Mu cts	From JM108 35
JM109	Δ(*pro-lac*) *thiA gyrA96 endA1 hsdR17 relA1 supE44 recA1*/F′ *traD36 proAB lacI*^{q} *ZΔM15*	35
M8820(Mu)	F⁻ Δ(*pro-lac*) Δ(*ara-leu*)*769 araD139 rpsL* Mu c^+	7
AB1133	F⁻ *thr-1 leuB6* Δ(*gpt-proA*)*62 hisG4 argE3 thi-1 lacY1 galK2 ara-14 xyl-5 mtl-1 rpsL31 supE44*	3
JC7623	AB1133 *tsx33 recB21 recC22 sbcB15*	33
JC7623*dapF*	JC7623 *dapF*::Mu *d*II PR13	This study
HfrH	*thi-1 rel-1*	25
KL14	Hfr *thi-1 rel-1*	3
KL14*dapF*	KL14 *dapF*::Mu *d*II PR13	This study
KL16	Hfr *thi-l rel-1*	25
PK191	Hfr Δ(*gpt-lac*) *thi-l rel-1*	25
MM383	*thyA36 lacZ53 rha15 polA12 rpsL151*	27
Gif106	*ilvA argH1000 metLM1000 thrA1101*	4
TP803	*met gal cya803 gyrA rpoB hsdR supE*	16
TP2006	F⁻ *lacΔX74 xyl cyaΔ*	16
Plasmids		
pHC79	Ap^r Tc^r derivative of pBR322 carrying the *cos* sequence of phage λ	17
pCR102	Ap^r derivative of pHC79 carrying the *dapF* gene	This study
pCR116	Ap^r derivative of pHC79 carrying the *dapF* gene	This study
pCR258	Ap^r derivative of pHC79 carrying the *dapF* gene	This study
pACYC184	Cm^r Tc^r	8
pUN121	Ap^r *tet* gene under the control of λ *c*I repressor	28
pDF1	Ap^r Tc^r *dapF*	This study
pDF2	Cm^r *dapF*	This study
pDF3	Ap^r *dapF*	This study
pDF31	Ap^r Cm^r *dapF*::Mu *d*II PR13	This study

Creation of a mutation in the chromosomal *dapF* gene. Plasmid pDF31 harboring a Mu *d*II PR13 insertion in the *dapF* gene was linearized with *Sma*I and used to transform strain JC7623, a *recB recC sbcB* strain that can be transformed by linear DNA (33). Exchange of the mutated copy of *dapF* with the chromosomal wild-type gene was selected for by plating transformed bacteria on LB plates in the presence of chloramphenicol (25 μg/ml) and DAP (20 μg/ml).

Pools of DAP and peptidoglycan precursors. Bacteria were grown at 37°C either in LB medium (25) or in 63 medium (25) supplemented with 0.4% glucose and the required amino acids. Cultures (1-liter) were rapidly chilled at A_{600} = 1 (about 2×10^{11} cells) and harvested. The extraction of free amino acids and peptidoglycan nucleotide precursors, as well as the analytical procedure used for their quantitation, was as previously described (23, 24). A Biotronik model LC2000 amino acid analyzer was used for the estimation of total DAP (LL plus *meso*) and for its isolation from the bacterial extract for further investigation.

Separation of DAP isomers. Although several techniques had been described for the separation of DAP isomers (19, 29), none but a recently published one (34) was truly satisfactory when small amounts of DAP or high-ratio values between isomers had to be measured. Pure samples of LL-DAP and *meso*-DAP were prepared previously in our laboratory (32). Bis(dimethylaminoazo)benzenesulfonyl derivatives of these DAP isomers were made by the method of Chang et al. (9) and subsequently separated and quantitated by reverse-phase high-pressure liquid chromatography on a Merck-Lichrosorb RP18 column (3.9 by 250 mm). Operating conditions were as follows: isocratic elution at 37°C was used with 12 mM ammonium phosphate (pH 6.5)–acetonitrile–dimethylformamide (69:27:4, vol/vol/vol) at a flow rate of 1 ml/min, with detection at 436 nm (Waters model 450 detector), and with a sensitivity of 0.01 absorbance units, full scale. Unpurified DAP preparations were similarly derivatized and analyzed, since DAP isomers were totally separated from other amino acid derivatives (see Fig. 2).

RESULTS

Cloning of the gene for DAP epimerase. Assuming that a strain harboring a plasmid carrying *dapF*, the structural gene for DAP epimerase, would overproduce this enzyme, we searched for such a clone in an *E. coli* genomic library constructed by Mechulam et al. (22) in the cosmid vector pHC79 (17). Among the 450 clones assayed, we found 3 that displayed 1.5- to 4.5-fold higher DAP epimerase activity than found in the host strain (Table 2). This overproduction factor is compatible with the low copy number of hybrid cosmids compared with that of pHC79 without insert (17).

Plasmids present in the three DAP epimerase-overproducing clones were subjected to restriction analysis by *Hin*dIII and *Bam*HI and were found to carry a common DNA region

TABLE 2. Overproduction of DAP epimerase in independent clones of the cosmid library

Strain and plasmid	DAP epimerase sp act	Overproduction factor[a]
IBPC111(pHC79)	71,430	1.0
IBPC111(pCR102)	113,130	1.6
IBPC111(pCR258)	140,310	2.0
IBPC111(pCR116)	319,110	4.5

[a] Ratio of DAP epimerase specific activity to that measured in IBPC111(pHC79).

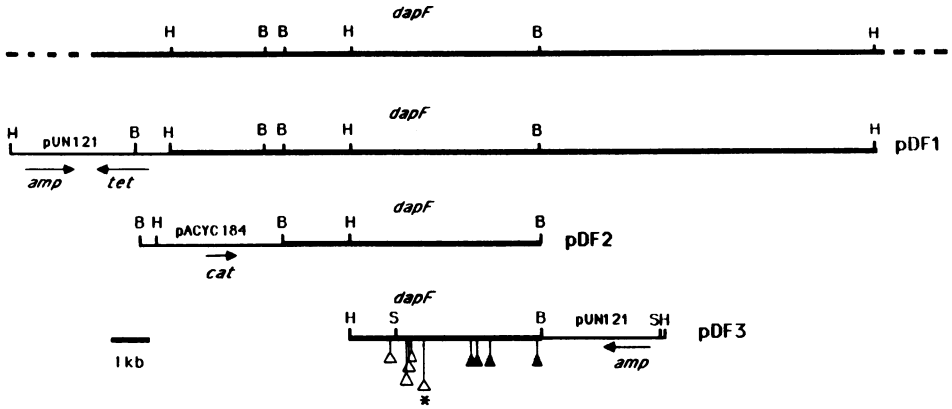

FIG. 1. Subcloning of the *dapF* gene. A *Hin*dIII (H) and *Bam*HI (B) restriction map of the common DNA region (heavy line) present in plasmids pCR102, pCR116, and pCR258 is shown. pDF1 was obtained by cloning a partial *Hin*dIII digest of pCR116 in pUN121 and selecting for ampicillin and tetracycline resistance in strain JM109. pDF2 was obtained by cloning a *Bam*HI digest of pCR116 in pACYC184 and selecting for chloramphenicol resistance in strain JM109. pDF3 was obtained after *Hin*dIII-*Bam*HI digestion of pDF1 and internal ligation, selecting in strain JM109 for ampicillin resistance. Insertional mutagenesis was performed on pDF3 by using transposon Mu *d*II PR13 to localize the *dapF* gene. Symbols: △, insertions inactivating *dapF* (including pDF31, *); ▲, insertions outside *dapF*. Owing to the presence of a *Hin*dIII site and a *Bam*HI site in Mu *d*II PR13, pDF31 was linearized by *Sma*I (S) before transforming strain JC7623 to transfer the *dapF*::Cm^r mutation onto the chromosome.

of at least 20 kilobases (kb) (Fig. 1). From cosmid pCR116, the smallest and the best DAP epimerase overproducer, various subclonings were performed with different vectors to reduce the size of the fragment carrying the *dapF* gene. Transformants were first selected according to their antibiotic resistance. Individual clones were then randomly screened for their DAP epimerase activity. Overproducing clones (6- to 10-fold, depending on the construction) were kept, and their plasmids were analyzed. These constructions are shown in Fig. 1 and detailed in its legend.

^{32}P-labeled plasmid pDF3 DNA was used as a probe to hybridize with chromosomal *E. coli* DNA digested with *Bam*HI and *Hin*dIII. The probe hybridized with a chromosomal fragment of the same size as the cloned fragment (5.2 kb), indicating that no gross rearrangement had occurred during the construction of the cosmid library (data not shown).

Localization of the cloned *dapF* gene by insertional mutagenesis. Insertional mutagenesis of plasmid pDF3 containing the *dapF* gene was performed by the in vivo method described by Castilho et al. (7) with the Mu *d*II PR13 bacteriophage derivative constructed by P. Ratet (Thèse 3e cycle). Of the Ap^r Cm^r transductants selected, 5 to 10% gave blue colonies on 5-bromo-4-chloro-3-indolyl-β-D-galactoside. A total of 28 Lac$^+$ Ap^r Cm^r transductants were analyzed for their DAP epimerase activity; 5 showed a chromosomal level of activity, while 23 retained the high level found with pDF3.

The five insertions inactivating the *dapF* gene were mapped within a 0.9-kb region (Fig. 1). Furthermore, these insertions were orientated according to the *lacZ* gene of Mu *d*II PR13 from the *Hin*dIII site toward the *Bam*HI site in the pDF3 insert, indicating that the *dapF* gene (translationally fused to the *lacZ* gene in these insertions) must be transcribed in that orientation.

Isolation of a chromosomal mutant with a mutation in *dapF*. The presence of Mu *d*II PR13 insertions in the *dapF* gene carried by plasmid pDF3 allowed us to perform reverse genetics by exchange of this mutation (leading to chloramphenicol resistance) with the wild-type chromosomal *dapF* gene. For this purpose, we used strain JC7623, which can be transformed with linear DNA (33). pDF31, one of the plasmids carrying a *dapF*::Mu *d*II PR13 inactivated gene, was restricted with *Sma*I and used to transform strain JC7623 to chloramphenicol resistance in the presence of DAP. The *Sma*I-generated fragment contained the selectable

TABLE 3. Pools of DAP and peptidoglycan precursors in wild-type and *dapF* strains[a]

DAP or peptidoglycan precursor	Amt (nmol/g [dry wt] of cell) in strain:			
	JC7623		JC7623*dapF*	
	LB medium	63 medium	LB medium	63 medium
LL-DAP	78.8	17.4	5,635	40,000
meso-DAP	82	17.4	56	ND[b]
UDP-*N*-acetylglucosamine	227	320	595	643
UDP-*N*-acetylmuramic acid	123	115	125	153
UDP-*N*-acetylmuramyl-L-Ala	4.7	3.8	4.7	3.9
UDP-*N*-acetylmuramyl-L-Ala-D-Glu	7.9	2.8	565	3.5
UDP-*N*-acetylmuramyl tripeptide	38	14	47	39
UDP-*N*-acetylmuramyl pentapeptide	677	610	800	1,730

[a] The doubling times were as follows: for JC7623 in LB medium, 47 min; for JC7623 in 63 medium, 74 min; for JC7623*dapF* in LB medium, 54 min; for JC7623*dapF* in 63 medium, 87 min.

[b] ND, Not determined. *meso*-DAP was detected but could not be measured with sufficient precision owing to the enormous ratio of LL- to *meso*-isomer.

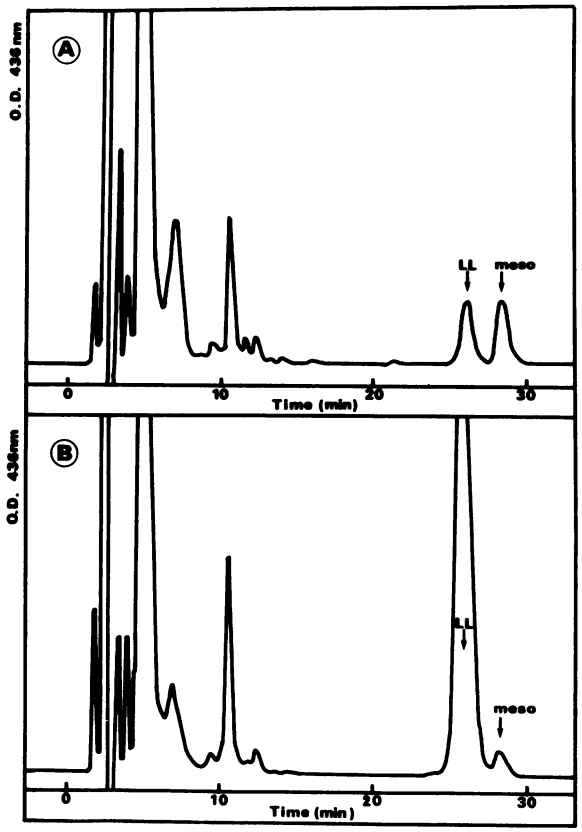

FIG. 2. Relative amounts of LL- and *meso*-DAP in bacteria. Aliquots from total DAP isolated from bacterial extracts were analyzed as detailed in Materials and Methods. (A) DAP isolated from strain JC7623; (B) DAP isolated from strain JC7623*dapF*. O.D. 436nm, Optical density at 436 nm.

marker Cm^r inserted in *dapF*, flanked by 0.8 and 3 kb of chromosomal DNA. This allowed integration of the Cm^r marker into the chromosome by a double-recombination event without acquisition of ampicillin resistance. A total of 75 clones were obtained after transformation of strain JC7623 with 2 μg of *Sma*I-cut pDF31. All of them were sensitive to ampicillin. Eleven were assayed and found to lack DAP epimerase activity (within the limits of precision of this assay. i.e., about 3% of the wild-type activity), confirming the disruption of the chromosomal *dapF* gene.

Characterization of the *dapF* mutant. To our surprise, lack of DAP epimerase activity in the *dapF* strain did not lead to a Dap^- phenotype, as this strain did not require the addition of *meso*-DAP for growth. Thus, pools of various intermediate metabolites were measured and are reported in Table 3. As expected, large amounts of LL-DAP were accumulated in the *dapF* strain (Fig. 2), but *meso*-DAP was still detected, while normal amounts of the DAP-containing peptidoglycan precursors (UDP-*N*-acetylmuramyl tripeptide and UDP-*N*-acetylmuramyl pentapeptide) (Fig. 3) were also found.

These results indicate that biosynthesis of *meso*-DAP still occurs in the *dapF* strain and explains the absence of a Dap^- phenotype. However, after growth in LB medium, the pool of *meso*-DAP was slightly decreased in the *dapF* strain that also accumulated large amounts of UDP-*N*-acetylmuramyl-L-Ala-D-Glu (Fig. 3; Table 3). These results suggest that *meso*-DAP biosynthesis in the *dapF* strain is limiting under these fast-growing conditions when, presumably, requirements for lysine and peptidoglycan biosynthesis are increased.

Such characteristics could be specific for this mutation, since a distal insertion in *dapF* could still lead to the synthesis of a partially active truncated protein. To address this question, the three central insertions located to the right of the *Sma*I site (Fig. 1) were similarly recombined onto the chromosome of strain JC7623. The three *dapF* strains obtained, although devoid of DAP epimerase activity, were still able to grow in the absence of DAP. Therefore, null mutations in the *dapF* gene do not lead to a Dap^- phenotype.

Genetic localization of the *dapF* gene. To localize the *dapF* gene on the *E. coli* chromosome, strain JC7623*dapF* was mated with different Hfr strains (HfrH, KL14, KL16, and PK191), and any loss of chloramphenicol resistance was monitored. This allowed us to map the *dapF* gene in the 80- to 100-min interval. The *dapF*::Cm^r mutation was then introduced by P1 transduction into strain KL14 (injecting in the clockwise direction from 68 min). This KL14*dapF* strain was used in interrupted mating with strain AB1133, and the Cm^r marker was found to enter about 3 min before *argE* (89.5 min). More accurate mapping was then performed by P1 cotransduction with several markers of this region. The *dapF*::Cm^r marker was not cotransducible with *argE* or with the *rha* locus (87.7 min) but was linked to *ilvA* (84.6 min) with a 70% frequency. As this region of the *E. coli* chromosome has already been well studied, we undertook a compilation of the available physical maps and found a good correlation between the map of the *dapF* region (Fig. 1) and that of the *hemC-metE* interval (10, 18, 31) that includes *cya* (20, 30), *uvrD* (13, 14, 21), *corA* (21), *pldA*, and *pldB* (12, 18), with the only exception being the rightmost *Hin*dIII site, which apparently belongs to the cosmid vector. These data (Fig. 4) indicate that the *dapF* gene was most probably located about 1 kb downstream of the *cya* gene, at 85 min. This was directly confirmed by checking for the presence of

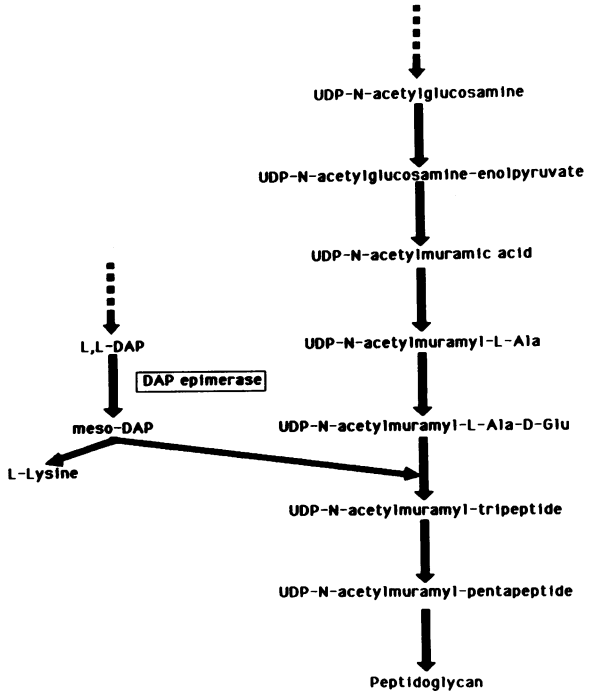

FIG. 3. Simplified biosynthetic pathway for peptidoglycan.

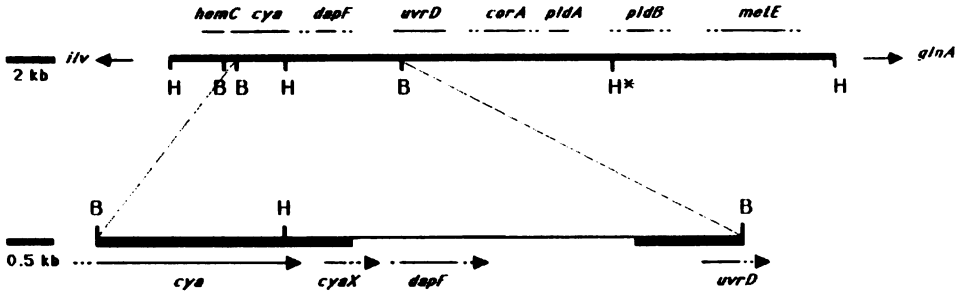

FIG. 4. Physical and genetic map of the *dapF* region. The location of the genes in the 85-min region of the *E. coli* chromosome has been correlated to a *Bam*HI (B)-*Hin*dIII (H) restriction map. H* indicates a site, probably absent from the chromosome (21), used for cloning of *dapF* (Fig. 1). An extension of the 7.3-kb *Bam*HI fragment shows the published DNA sequences around *dap*F (heavy bars) (1, 14) and its approximate position as defined by Mu *d*II PR13 insertions.

a complete *cya* gene in plasmid pDF1 (which was actually found to restore a sugar-utilizing phenotype in strains TP803 and TP2006); plasmid pDF2 (which should carry only the distal part of the *cya* gene) did not correct the *cya* defect of these strains.

DISCUSSION

In *E. coli*, *meso*-DAP, the precursor of lysine and a constituent of peptidoglycan, is synthesized through isomerisation of LL-DAP by DAP epimerase. We have identified the gene coding for this enzyme, *dapF*, in a cosmid library by screening for clones with amplified DAP epimerase activity. Insertional mutagenesis with Mu *d*II PR13 led to a precise localization of the *dapF* gene on the cloned fragment and, furthermore, tagged this gene with a selectable marker that allowed subsequent inactivation of the chromosomal *dapF* gene. No mutation in the *dapF* gene has been described before which can now been explained, since a *dapF* mutant strain does not require DAP for growth even in minimal medium, contrary to all the *dap* strains mutated in the other enzymatic steps (6). The metabolic block of the *dapF* strain was confirmed by an important accumulation of LL-DAP; however, *meso*-DAP was still present, a result which correlates well with the Dap^+ phenotype. These results could be explained if the so-called *dapF* gene was actually encoding a regulatory protein inducing the synthesis or stimulating the activity of DAP epimerase. This possibility is now excluded, since the N-terminal sequence of purified DAP epimerase (Met-Gln-Phe-Ser-Lys) (M. Bruschi and J. Bonicel, personal communication) fits perfectly with the nucleotide sequence of the gene located downstream of *cyaX* (A. Roy and A. Danchin, personal communication).

How is *meso*-DAP synthesized in the absence of a functional *dapF* gene? A plausible model is the existence of a second DAP epimerase, this other activity being undetectable in vitro under our experimental conditions. Duplication of genes encoding DAP epimerase would be in accordance with the dual function of this enzyme, necessary for both protein and peptidoglycan biosynthesis. However, the large accumulation of LL-DAP observed in the *dapF* strain indicates that this second enzyme should not be very active and might play quite a secondary role in wild-type bacteria. Alternatively, the high internal concentration of LL-DAP in the *dapF* strain could induce the synthesis of a *meso*-DAP dehydrogenase. Such an enzyme, which converts tetrahydrodipicolinate to *meso*-DAP in a single step, has been found in some bacteria but not in *E. coli* (26), in which it might be cryptic under normal conditions. However, we favor the hypothesis that LL-DAP is converted to *meso*-DAP in the *dapF* strain by some other amino acid racemase, acting nonspecifically and with a low efficiency in nonphysiological concentrations of LL-DAP (which can reach 10 mM, as calculated from Table 3).

Genetic mapping experiments have localized the *dapF* gene around 85 min on the *E. coli* chromosome. This result was then improved by direct correlation with the physical map of this region. The *dapF* location is now known within a few hundred base pairs and coincides with that of a gene encoding a 32-kilodalton polypeptide (20), while DAP epimerase has been shown to have an active monomer of 34 kilodaltons (34). The minimum extent of the *dapF* gene (as defined by Mu *d*II PR13 insertions) localizes the beginning of *dapF* about 1 kb downstream of the *Hin*dIII site in *cya* (Fig. 4). All the genes identified in that region, *cya*, *cyaX*, *dapF*, and *uvrD*, are transcribed in the clockwise direction, and some of them could be transcribed as polycistronic mRNAs. Sequencing and transcriptional mapping will address this question.

Identification of the *dapF* gene, besides contributing to the understanding of the organization of the 85-min region of the *E. coli* chromosome, opens the way to the study of another gene involved in lysine-DAP biosynthesis. This new locus is not linked to the seven others.

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