Two Genes Involved in the 1,3-Diaminopropane Production Pathway in *Haemophilus influenzae*

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We previously cloned and sequenced the *Acinetobacter baumannii* *dat* and *ddc* genes encoding L-2,4-diaminobutyrate: 2-ketoglutarate 4-aminotransferase (DABA AT) and DABA decarboxylase, respectively, involved in the 1,3-diaminopropane (DAP) production pathway. Homology searches of the gene products provided an indication that a similar gene cluster is present in the genome of *Haemophilus influenzae* Rd. This was first verified by detection of the corresponding enzyme activities in and the production of DAP by all *H. influenzae* strains examined. Both of the crude enzymes from the representative strain of *H. influenzae* showed catalytic properties essentially similar to the *A. baumannii* DABA AT and DABA DC. An *Escherichia coli* clone carrying the *dat* homolog of *H. influenzae* Rd showed a high level of DABA AT activity, the enzyme protein responsible being detected by immunoblot analysis. These results specify the two *H. influenzae* genes involved in DAP production.

Key words 1,3-diaminopropane synthesis; *Haemophilus influenzae*; *Acinetobacter baumannii*

Haemophilus influenzae is responsible for many human infections, including otitis media, meningitis, epiglottitis and pneumonia, and it is recognized that members of serotype b are most likely to cause invasive infections.¹⁾ Recently, Fleischmann *et al.*²⁾ have reported the 1.83 Mb complete genome sequence of *H. influenzae* Rd, in which a total of 1743 predicted protein genes were identified. Moreover, computer analyses by others^{3,4)} revealed the presence of a number of additional genes encoding putative proteins, 83% of the gene products being functionally predicted. However, proposed protein functions are, for the most part, implied from the known functions of related proteins of *Escherichia coli*, and it is unclear that functional predictions for the respective genes, particularly those with low homology, are correct. In this context, observed high sequence homologies of the *H. influenzae* genes with other characterized genes of known functions in microorganisms other than *E. coli* are also expected to provide information about yet unknown biochemical pathways in this species, and in such cases functional clarification of the relevant gene products will aid in better understanding the molecular and evolutionary bases for biochemical and physiological processes in *H. influenzae*.

We previously reported the two *Acinetobacter baumannii* genes, *dat* and *ddc*, encoding novel enzymes, L-2,4-diaminobutyrate: 2-ketoglutarate 4-aminotransferase (DABA AT)⁵⁾ and DABA decarboxylase (DC),⁶⁾ respectively, involved in the pathway of 1,3-diaminopropane (DAP) synthesis. These genes are organized in a typical operon structure, and are not present in *E. coli*. In this study, we show that the two *H. influenzae* genes, HI0949 and HI0946.1, are highly homologous in their deduced amino acid sequences to *A. baumannii* *dat* and *ddc* genes, and demonstrate that *H. influenzae* strains actually express these enzyme activities with the production of DAP.

MATERIALS AND METHODS

Bacterial Strains and Culture Conditions *H. influenzae* types a, b, d, e and f (Table 1) were obtained from the Laboratory Culture Collection, the Institute of Medical Science, The University of Tokyo (Tokyo) through the Japanese

Society for Bacteriology, and *E. coli* clones, ATCC 612778 and ATCC 615394, which carry pUC18-derived plasmids GHIFZ90 and GHIHB19, respectively, were from the American Type Culture Collection. The plasmids GHIFZ90 and GHIHB19 contain the *H. influenzae* Rd HI0949 and HI0946.1 genes, respectively. *H. influenzae* strains were cultivated on chocolate agar plates (Nissui, Tokyo) at 37 °C for 48 h, cells were collected with 0.5% NaCl containing 10 mM MgCl₂ (pH 7.2) and the suspension was centrifuged at 4000×g for 20 min to obtain a cell pellet. *E. coli* ATCC 612778 and 615394 were grown in LB medium containing ampicillin (50 mg ml⁻¹) at 37 °C for 8 h, and cells were harvested by centrifugation. *A. baumannii* ATCC 19606 was grown as described previously.⁷⁾

Di- and Polyamine Determination Cells (ca. 300 mg wet weight) were suspended in 20 ml of 2% HClO₄ and then disrupted by sonication, and the whole supernatant after centrifugation was used for determination of di- and polyamines by gas-liquid chromatography (GLC).⁸⁾

Enzyme Assays Cells were suspended in 5 volumes of 20 mM Tris-HCl, pH 7.5, containing 0.1 mM EDTA and 40 mM pyridoxal phosphate (buffer A), and sonicated on ice. Cell debris was removed by centrifugation for 30 min at 40000×g, and the supernatant was dialyzed for 12 h at 4 °C against buffer A for enzyme assay. In some experiments, the supernatant without dialysis was fractionated with ammonium sulfate, and the precipitates (40–60% and 60–75% saturation) were used after dialysis against buffer A. DABA AT and DABA DC activities were measured as described previously.^{5,7)} Protein was determined by the Lowry method with bovine serum albumin (BSA) as the standard.

Other Methods Homology searches and alignments were performed with GenBank databases⁹⁾ and the Genetyx-Mac software (Software Development, Tokyo), respectively. Cell extracts from various strains of *H. influenzae* were boiled for 5 min before use in electrophoresis. Proteins were separated on an sodium dodecyl sulfate (SDS)-10% polyacrylamide gel and transferred to a Hybond-C membrane (Amersham) with a Trans-Blot SD electrophoretic blotting cell (BioRad). Immunoreactive proteins were visualized by sequential incubation with a 1:500 dilution of rabbit poly-

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clonal antibodies against the *A. baumannii* DABA AT⁵⁾ or DABA DC⁶⁾ and a 1:2000 dilution of goat anti-rabbit IgG conjugated with alkaline phosphatase (Boehringer Mannheim) as described.⁷⁾

RESULTS AND DISCUSSION

Deduced Amino Acid Sequences of *H. influenzae* Genes Homologous to *A. baumannii* *dat* and *ddc* Comparison of the deduced amino acid sequence of the *A. baumannii* *ddc* gene (GenBank accession number D55724) with other protein sequences in the GenBank revealed the presence of a gene with 76% overall identity (88% similarity) in the intergenic region of the *H. influenzae* Rd genes, HI0946 and HI0947. This open reading frame had not been listed in the report of Fleischmann *et al.*,²⁾ but is now deposited as HI0946.1 (U32776). Further homology searches identified in a region upstream of HI0946.1 the gene HI0949 (U32776) with 70% overall identity (92% similarity) to the *A. baumannii* *dat* gene (AB001599). Since the HI0949 gene product shared a similarity with *E. coli* γ -aminobutyrate aminotrans-

ferase (34% identity), the gene was tentatively assigned as *gabT*.²⁾ The lysine residues, putative pyridoxal phosphate binding sites, of the deduced amino acid sequences of two *H. influenzae* genes (K-286 in HI0949 and K-320 in HI0946.1) are each aligned to the equivalent positions of the *A. baumannii* DABA AT and DABA DC (Fig. 1). Moreover, the molecular masses predicted from the deduced amino acid sequences of HI0949 (49.4 kDa) and HI0946.1 (56.2 kDa) are comparable to those of the *dat* (47.4 kDa) and *ddc* products (56.2 kDa), respectively. While the *A. baumannii* *dat* and *ddc* genes are separated only by 2 bp constituting an operon, the two *H. influenzae* Rd genes are interrupted by two open reading frames (ca. 800 bp) (Fig. 2), whose functions are not clear. Although the functions of the two genes highly homologous to *dat* and *ddc* are totally unknown in *H. influenzae*, these results provided an indication that they encode the enzymes involved in the pathway for DAP production.

Production of DAP by *H. influenzae* Since *H. influenzae* showed only poor growth in media devoid of polyamines, we used the chocolate agar plate which had been found to contain small amounts of putrescine, spermidine and sper-

(A)

A. b. <i>dat</i>	1:MSVTSVNPATNATNEYLLTQSQMESNVRSYPRKLPALAIKAQGCWVTDVEGTQYLDCLAGAGTLALGHNHPAVIQSIQD	80
H. i. HI0949	1:MTMITPVQAILASNQHFLLDRQDVMSNVRSYPRKLPFAYAKAQGCWVTDVEGNEYLDLFLAGAGTLALGHNHPILMQAIKD	80
	* * * * *	
A. b. <i>dat</i>	81:TLASGLPLHTLDLTPLKDAFTTEALLAYLPGGKEEYCLQFCGPGADATEAAIKLAKTYTGRSSVISFSGGYHGMTHGSL	160
H. i. HI0949	81:VLDLGLPLHTLDLTPLKDAFSEILLSFFP--KDKYILQFTGPGADANEAAIKLAKTYTGRGNIIFSGGFGHMTQAGAL	158
	* * * * *	
A. b. <i>dat</i>	161:AMTGNLSAKNAVNGLMPGVQFMPYPHEYRCPLGLGGEAGVDALTYFFENFIEDVESGVTKPAAVILEAIQEGGVVTA	240
H. i. HI0949	159:ALTGNLGAKNVENLMPGVQFMPYPHEYRCPLGLGGEAGVQYFFENFIEDVESGVVTKPAAVILEAIQEGGVVSA	238
	* * * * *	
A. b. <i>dat</i>	241:KWLQKIREVTEKHNIIVLILDEVQAGFARSGKMFAPFHAGIEPDIVVMSKAVGGGLPLAVLGIRKFDAPQAGHTGTFRG	320
H. i. HI0949	239:SFLQKVREVTQKHGILMIVDEVQAGFCRSGRMFAFEHAGIEPDIIVMSKAVGGSLPLAVLAIRKEFDAPQAGHTGTFRG	318
	* * * * *	
A. b. <i>dat</i>	321:NQLAMGTGLVLETIKEQNLQAQNERG-----EFPICIGNVRGRGLMIGVEIVDERKPADRIGSHPADSQLAA	388
H. i. HI0949	319:NQLAMATGYASLKMIRDENLAQNAQERGEYLTNALRELSKEYPCIGNVRGRGLMGIDIVDERQSKDATGAYPRDCELAA	398
	* * * * *	
A. b. <i>dat</i>	389:AIQTACFNNNLLLEKGRNGTVIRLLCPLIITQEECEVEIARFKKAVAEALVAVRGA	445
H. i. HI0949	399:AIQKACFKNKLLERGGNGNVVRLCAVNINQSECEEFIKRFQSVVDALKVVR-	454
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(B)

A. b. <i>ddc</i>	1:MVDFAEHRKALLCNDQAQSIADYESAMGEAVKAVSAWLQNEKMYTGGSIKELRSAIS-FQPSKEGGMVQOQSLQRMIELFLN	79
H. i. HI0946.1	1:MSNLKQKQALFCNDNEAINDYETAMHNAVQAVSAWLQNEKMYTGGSIKQMRALISGFNPTKEGGMVQKSLDHLVEIFLN	80
	* * * * *	
A. b. <i>ddc</i>	80:KSLKVHHPHSLAHLHCPTMVMSQIAEVLINATNQSMDSWDQSPAGSLMEVQLIDWLRLQKVGYSQAGVFTSGGTQSNLM	159
H. i. HI0946.1	81:PSLKVVHHPHSLAHLHCPTMVTSQIAEVLINATNQSMDSWDQSPAGSIMEEHLINWLRLQKAGYGEFTSGGTQSNLM	160
	* * * * *	
A. b. <i>ddc</i>	160:GVLLARDWCIAKNWKDENGPNVSVQDGIAPAEAMKNVVKICSENAHFSVQKNMAMMGMGFQSVVTVPVNENAOQMDVDALE	239
H. i. HI0946.1	161:GVLLARDWAIANHWKNEGSEWSVQDGIAPAEAMQKVVVKICSENAHFSVQKNMAMMGMGFQSVVTVPSNANAOQMDLIAK	240
	* * * * *	
A. b. <i>ddc</i>	240:KTMHLQAEQKVVACVATAGTTDAGAIHPLKKIREITNKYGSWMHIDAAGGALLSNTYRAMLDGIELSDSITLDFH	319
H. i. HI0946.1	241:QTLAQLKADGKITACIVATAGTTDAGATDDLKAIKRLADEYQAWLHVDAAGGALLSKDYRYFLDGIELTDSITLDFH	320
	* * * * *	
A. b. <i>ddc</i>	320:HYFQISCGAFLKDEANRYRFMYEAYELNSAYDEEHGVPNLVSKSLQTTTRFDALKLWMTIESLGEELYSGMIDHGVKL	399
H. i. HI0946.1	321:HFFQITISCGAFLKDPENYRFIDYKADYLNSEYDEAHGVPNLVAKSLQTTTRFDALKLWFTLEALGEDLYASMIDHGVKL	400
	* * * * *	
A. b. <i>ddc</i>	400:TREVADYIKATEGLELLVEPQFASVLFVRVPEGYPVEFIDSLNQNADELFAERGEANIGTVKGVNQSLKMTTLSPVTV	479
H. i. HI0946.1	401:TKEVEQYINDTPDLEMLVPSQFASVLFVRVPEKDYPAEFIDALNQNADELFAERGEANIGTVKGVNQSLKMTTLSPVTV	480
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A. b. <i>ddc</i>	480:DNVKNLLAQVLAEEAERIKDAIASGNYVPPID	510
H. i. HI0946.1	481:ENVKALLTQVLTANRIKDDIKNGTYTPPID	511
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Fig. 1. Alignments of the Deduced Amino Acid Sequences of *A. baumannii* *dat* and HI0949 (A) and Those of *A. baumannii* *ddc* and HI0946.1 (B)

Identical residues are indicated by asterisks, and gaps, indicated by dashes, were introduced to allow for an optimal alignment. The K residue, a putative pyridoxal phosphate binding site, is boxed.

mine but no DAP. DAP was detected at appreciable amounts in cell lysates from all strains of *H. influenzae* examined (Table 1) together with other di- and polyamines present in the agar plate (data not shown). Identity of DAP was confirmed by GC-MS (data not shown). Some bacterial species have been reported to have the spermidine dehydrogenase or spermidine oxidase giving rise to DAP.^{10,11} Therefore, cell lysates were incubated under the same conditions as those of Okada *et al.*¹⁰ and Smith,¹¹ and the products, DAP and Δ^1 -pyrroline, were determined by the present GLC method and the colorimetric method,¹¹ respectively. However, no such activity was found in any *H. influenzae* strain tested, consistent with the results obtained for *Acinetobacter* species.¹²

Detection of DABA AT and DABA DC Activities in *H. influenzae* To clarify the existence of DABA AT and DABA DC activities in *H. influenzae*, crude cell lysates were first assayed under the same conditions as used for *A. baumannii*.^{5,7} All strains examined showed both enzyme activities, as summarized in Table 1. Removal of pyridoxal phosphate from the assay mixture resulted in almost complete disappearance of both enzyme activities, indicating their pyridoxal phosphate-dependence. Next, to examine some properties of the enzymes, a cell lysate from *H. influenzae* IID 984 was fractionated with ammonium sulfate. DABA AT activity was detected in the precipitate of 40–60% saturation, and DABA DC in the precipitate of 60–75% saturation, in which no ornithine or lysine decarboxylase activities were detected. When 2-ketoglutarate was used as an amino group acceptor, the highest activity was observed for L-DABA; γ -aminobutyric acid, ornithine and lysine as amino group donors showed 28, 7 and 14% activity, respectively, relative to L-DABA. These results were very similar to those observed with the DABA AT from *A. baumannii*.⁵ On the other hand, the decarboxylase activity with high substrate specificity towards L-DABA was enhanced *ca.* 8-fold and 5-fold, respectively, by the addition of 2.5 mM Ca^{2+} and Mg^{2+} to the

Fig. 2. Arrangement of the Genes Relevant to DAP Production in *A. acinetobacter* ATCC 19606 and *H. influenzae* Rd

The gene arrangement in *H. influenzae* Rd was constructed based on the data provided by the TIGR Internet server (<http://www.tigr.org>).

Table 1. DAP Content and DABA AT and DABA DC Activities in Some Strains of *H. influenzae*^{a)}

Strains	DAP (nmol/g wet weight cells)	Specific activity	
		DABA AT ^{b)}	DABA DC ^{c)}
<i>H. influenzae</i> IID 983 (= ATCC 9327, type a)	170	265	268
IID 984 (= ATCC 9334, type b)	180	344	851
IID 986 (= ATCC 9332, type d)	297	386	367
IID 987 (= ATCC 8142, type e)	264	324	394
IID 988 (= ATCC 9833, type f)	110	185	44

a) All organisms were grown on chocolate agar plates for 48 h at 37 °C. DAP and the enzyme activities were measured as described in Materials and Methods. b) nmol glutamic acid formed/mg protein/h. c) nmol DAP formed/mg protein/h.

assay mixture, as compared with no addition; this was consistent with the finding that activation by these cations is one of the most remarkable features of the *Acinetobacter* DABA DC.⁷⁾

An attempt was made with an immunoblot technique to detect the DABA AT and DABA DC proteins in the cell lysates of *H. influenzae*. A band weakly cross-reactive with the antiserum against the *A. baumannii* DABA AT was found in strain IID 986 (type d), which had a somewhat higher M_r than that of *A. baumannii* (Fig. 3). The M_r of this band corresponds with that calculated from the deduced amino acid sequence of the *H. influenzae* Rd (capsule-deficient type d¹³) HI0949 gene. Therefore, the cross-reactive proteins in the 47–50 kDa range seen in other lanes may be homologs of DABA AT, although the staining intensity of the proteins and their M_s were inconsistent with each other. In contrast, no band cross-reactive with anti-*A. baumannii* DABA DC antiserum was seen in the region, where the enzyme protein should have migrated in SDS-polyacrylamide gel electrophoresis (PAGE). This may be due to difference in antigenicity, but not to the contents of the enzyme proteins to be tested, since all strains except for IID988 also showed DABA DC activity at levels similar to those of *Acinetobacter* species.

Expression of the *H. influenzae* Rd Genes in *E. coli*

Finally, to specify the genes relevant to DAP production in *H. influenzae*, *E. coli* clones of the *H. influenzae* Rd genome in the TIGR/ATCC Microbial Special Collection were analyzed for the DABA AT and DABA DC activities. *E. coli* ATCC 612778 showed a high level of DABA AT activity (17.3 $\mu\text{mol/mg protein/h}$), which was comparable to that ob-

Fig. 3. Immunoblot Analysis of Cell Lysates of *H. influenzae* Probed with Antibodies against *A. baumannii* DABA AT

Each slot received 50 μg of protein. Lane 1, molecular mass standards in kDa; lane 2, *A. baumannii* ATCC 19606 as a positive control for DABA AT (47.4 kDa); lanes 3–7, *H. influenzae* IID 983, IID 984, IID986, IID 987 and IID 988, in that order. The arrow indicates the expected DABA AT proteins in *H. influenzae* strains. Bands seen in the upper part of the gel are probably non-specific.

Fig. 4. Immunoblot Analysis of the Cell Lysate of *E. coli* ATCC 162778 Probed with Antibodies against *A. baumannii* DABA AT

Each slot received 50 μ g of protein in the cell lysate. Lane 1, molecular mass standards in kDa; lane 2, *A. baumannii* ATCC 19606; lane 3, *E. coli* ATCC 612778. The arrow indicates the DABA AT overexpressed in *E. coli*.

served when the *A. baumannii* *dat* gene was expressed in *E. coli*.⁵⁾ Consistent with this result, the protein band cross-reactive with the antiserum was clearly detected by immunoblotting (Fig. 4). Unfortunately, *E. coli* clone ATCC 615394, although expected to contain the corresponding gene, failed to exhibit the DABA DC activity, suggesting that the cloned fragment probably lacks part of the gene required for its expression.

In conclusion, the results presented here show that *H. influenzae* has the pathway for the production of DAP, in which the two genes highly homologous to the *A. baumannii* *dat* and *ddc* genes are involved. From an evolutionary point of view, it is of interest that *H. influenzae* has some genetic relations with *Acinetobacter* species, although the biological significance of DAP in these species is unknown.

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