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The Ornithine Decarboxylase Gene *odc* Is Required for Alcaligin Siderophore Biosynthesis in *Bordetella* spp.: Putrescine Is a Precursor of Alcaligin

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Chromosomal insertions defining *Bordetella bronchiseptica* siderophore phenotypic complementation group III mutants BRM3 and BRM5 were found to reside approximately 200 to 300 bp apart by restriction mapping of cloned genomic regions associated with the insertion markers. DNA hybridization analysis using *B. bronchiseptica* genomic DNA sequences flanking the cloned BRM3 insertion marker identified homologous *Bordetella pertussis* UT25 cosmids that complemented the siderophore biosynthesis defect of the group III *B. bronchiseptica* mutants. Subcloning and complementation analysis localized the complementing activity to a 2.8-kb *B. pertussis* genomic DNA region. Nucleotide sequencing identified an open reading frame predicted to encode a polypeptide exhibiting strong similarity at the primary amino acid level with several pyridoxal phosphate-dependent amino acid decarboxylases. Alcaligin production was fully restored to group III mutants by supplementation of iron-depleted culture media with putrescine (1,4-diaminobutane), consistent with defects in an ornithine decarboxylase activity required for alcaligin siderophore biosynthesis. Concordantly, the alcaligin biosynthesis defect of BRM3 was functionally complemented by the heterologous *Escherichia coli speC* gene encoding an ornithine decarboxylase activity. Enzyme assays confirmed that group III *B. bronchiseptica* siderophore-deficient mutants lack an ornithine decarboxylase activity required for the biosynthesis of alcaligin. Siderophore production by an analogous mutant of *B. pertussis* constructed by allelic exchange was undetectable. We propose the designation *odc* for the gene defined by these mutations that abrogate alcaligin siderophore production. Putrescine is an essential precursor of alcaligin in *Bordetella* spp.

Nutritional iron limitation, mediated primarily by specific host iron-binding glycoproteins, is a front-line host defense against disease-causing infectious agents. Strategies aimed at defeating host iron restriction may involve the action of low-molecular-mass, high-affinity, ferric iron-specific chelators of microbial origin, termed siderophores, that are synthesized coordinately with their cognate surface receptors and transport machinery in response to iron starvation (28). The role of siderophores in microbial pathogenesis is well established (29, 53, 54).

Bordetella pertussis, the causative agent of human whooping cough or pertussis, and *Bordetella bronchiseptica*, the agent of swine atrophic rhinitis and kennel cough in dogs, are mucosal pathogens that colonize the upper respiratory tracts of their mammalian hosts. In the first reported molecular genetic studies of *Bordetella* iron acquisition systems, Armstrong and Clements (3) described the isolation of *B. bronchiseptica* mutants deficient in siderophore activity following transposon mutagenesis. DNA hybridization analysis using DNA probe sequences flanking the transposon insertions established the existence of homologs of *B. bronchiseptica* siderophore genes in *B. pertussis*. Reciprocal cross-feeding experiments provided additional support for the hypothesis that the hydroxamate siderophores produced and utilized by *B. pertussis* and *B. bronchiseptica* were structurally similar or identical. In a subsequent report, complementation of deregulated iron transport system mutants of *B. bronchiseptica* by the *fur* gene of *B. pertussis* (9) confirmed the involvement of common regulatory determinants governing iron transport in the two related species.

Recent purification and spectroscopic analysis of *Bordetella* siderophores (10, 33) found that the iron chelators produced by both *B. pertussis* and *B. bronchiseptica* were identical to the potent macrocyclic dihydroxamate siderophore alcaligin, 1,8(*S*), 11,18(*S*)-tetrahydroxy-1,6,11,16-tetraazacycloeicosane-2,5,12, 15-tetrone (molecular formula, $C_{16}H_{28}N_4O_8$; molecular weight, 404), previously isolated from the taxonomically related bacterial species *Alcaligenes denitrificans* subsp. *xylosoxydans* (35, 36). Evidence for biological activity of the purified siderophores was gathered with growth stimulation and ^{55}Fe-alcaligin transport assays of *Bordetella* spp. (10). Uptake rates and saturability of iron uptake observed in ^{55}Fe transport assays were indicative of a high-affinity ferric alcaligin transport system and provided the first direct evidence, beyond simple growth stimulation assays, of alcaligin-mediated *Bordetella* iron transport.

We report the identification of a gene encoding an alcaligin siderophore biosynthetic activity defined by previously reported transposon mutations abrogating alcaligin production in *B. bronchiseptica* (3) as an ornithine decarboxylase (Odc) gene, indicating that putrescine is an essential precursor for alcaligin siderophore biosynthesis in *Bordetella* spp.

MATERIALS AND METHODS

Bacterial strains and plasmids. *B. bronchiseptica* B013N, a nalidixic acid-resistant derivative of strain B013, and the isolation of siderophore biosynthetic mutants BRM3 and BRM5 by mini-Tn*5*/*lacZ1* (16) insertional inactivation have been described previously (3). Virulent-phase *B. pertussis* UT25 has also been described previously (18). *Escherichia coli* DH5α [F$^-$ F80d*lacZ*ΔM15 Δ(*lacZYA-argF*)U169 *endA1 recA1 hsdR17*(r_K^- m_K^+) *deoR thi-1 supE44* λ$^-$ *gyrA96 relA1*]

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(Bethesda Research Laboratories, Gaithersburg, Md.) was used as the host strain for routine plasmid construction and propagation and as the donor strain in conjugal transfer of plasmids to *Bordetella* strains. Plasmid pRK/3.2P, carrying the *speC* constitutive ornithine decarboxylase gene of *E. coli*, was constructed by subcloning the 3.2-kb *E. coli Pst*I insert DNA fragment of plasmid pODC1 (8) into the broad-host-range plasmid vector pRK415 (26). DH5α (pRK2013) was used as the source of plasmid-encoded mobilization functions (19) in triparental matings to transfer the cosmid pCP13-based gene bank of *B. pertussis* UT25 (11) as well as plasmid vector pRK415 derivatives to *Bordetella* strains. *E. coli* K38 (pGP1-2) (46) was used as the host strain in *Bordetella* protein expression studies.

Growth conditions. *B. pertussis* was maintained on Bordet-Gengou agar plates (7), and *B. bronchiseptica* was maintained on blood agar plates or standard Luria-Bertani agar plates. Modified Stainer-Scholte medium (39, 41) (SS) was used for broth cultures of *Bordetella* spp. Iron-replete and iron-depleted SS culture conditions were achieved by the methods of Armstrong and Clements (3); media and supplements were deferrated with Chelex-100 resin (Bio-Rad Laboratories, Richmond, Calif.). Tetracycline was used at 15 μg/ml to select for pCP13-based cosmids and pRK415 plasmid derivatives. Kanamycin was used at 50 μg/ml for maintenance of pRK2013 and pGP1-2 and for selection of kanamycin cassette insertion markers in allelic exchange procedures, while ampicillin was used at 100 μg/ml for maintenance of other plasmids in *E. coli*. Culture media were supplemented as required with cadaverine, agmatine, putrescine, ornithine, arginine, or spermidine at a 50-μg/ml final concentration, or with 1,4-diamino-2-butanone dihydrochloride (DAB) (Sigma Chemical Co., St. Louis, Mo.) at concentrations ranging from 0 to 2 mM. All glassware was acid cleaned and rinsed repeatedly in distilled deionized water prior to use. Optical densities of SS cultures were monitored with a Klett-Summerson colorimeter fitted with a no. 54 filter (Klett Manufacturing Co., Long Island City, N.Y.).

Conjugation. Conjugal transfer of pCP13 and pRK415 derivatives to *Bordetella* strains was accomplished by triparental matings with *E. coli* DH5α as the plasmid donor strain and DH5α (pRK2013) as the source of mobilization functions. Suspensions of donor, helper, and recipient strains in SS plus 10 mM $MgSO_4$ were combined at an estimated 1:1:3 cell ratio, spotted onto Luria-Bertani or blood agar plates (for *B. bronchiseptica* recipients) or Bordet-Gengou agar plates (for *B. pertussis* recipients), and incubated at 37°C for 3 to 5 h. Bacteria were streaked from mating spots directly onto agar plates containing the appropriate selective antibiotics and crude colicin B (3, 12). Plates were incubated at 37°C for 1 to 4 days, at which time transconjugant colonies were apparent.

Routine DNA procedures. DNA cloning and hybridization analysis were performed as described previously (38), and transformation of *E. coli* was by the $CaCl_2$ method of Cohen and coworkers (14).

Radiochemicals. Tran ^{35}S-label used for radiolabeling plasmid-encoded *B. pertussis* proteins in *E. coli*, [^{32}P]dATP for nucleotide sequencing, and [^{32}P]dCTP for DNA hybridization analysis were purchased from ICN Radiochemicals (Irvine, Calif.). L-[1-^{14}C]ornithine used in Odc assays was obtained from NEN Research Products (Wilmington, Del.).

Conditional expression of plasmid-encoded *Bordetella* proteins in *E. coli*. Exclusive expression of *Bordetella* proteins in *E. coli* used the temperature-sensitive T7 RNA polymerase-promoter system of Tabor and Richardson (46). Recombinant plasmid pT7/*odc* carries an approximately 2.8-kb *odc*$^+$ DNA fragment of *B. pertussis* UT25 in the T7 promoter plasmid vector pGEM4Z (Promega Corporation, Madison, Wis.). Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis of radiolabeled bacterial cell proteins was performed essentially as described previously (27).

Nucleotide sequencing. Nucleotide sequencing was performed by the dideoxy chain termination method with a Sequenase version 2.0 kit (United States Biochemical Corp., Cleveland, Ohio). Double-stranded plasmid templates were isolated by the alkaline lysis procedure (6) and further purified by precipitation in the presence of 500 mM sodium chloride and 6.5% polyethylene glycol 8000. Nucleotide sequence data were derived from plasmid subclones of *odc*$^+$ *B. pertussis* cosmids, as well as from a collection of deletion derivatives generated by treatment of *odc*$^+$ plasmid subclones with exonuclease III (21) by using a commercially available kit (Erase-a-Base; Promega Corporation) or from deletion derivatives resulting from in vivo intramolecular transposition of transposon γδ (52) with the Deletion Factory System version 2.0 (Life Technologies, Inc., Gaithersburg, Md.). Nucleotide sequence data management and analysis used DNA Strider 1.2 software (Service de Biochimie et de Génétique Moléculaire, Gif sur Yvette, France) for the Macintosh computer. Database searches and data retrievals used the BLAST (1) and RETRIEVE electronic mail servers developed by the National Center for Biotechnology Information at the National Library of Medicine. For protein database BLASTP searches, *Bordetella* DNA sequences were translated in all six possible reading frames, and the resulting amino acid sequences were transmitted to the National Center for Biotechnology Information for analysis. Multiple amino acid sequence alignments were performed with the MegAlign module of a demonstration version of the Lasergene sequence analysis software system for the Power Macintosh computer (DNA STAR, Inc., Madison, Wis.).

Siderophore assays. Chrome azurol S (CAS) agar plates for scoring of *B. bronchiseptica* siderophore production were prepared as described by Armstrong and Clements (3). The CAS universal siderophore detection assay (40) was used to monitor siderophore production by *Bordetella* strains grown in liquid culture by measuring the decrease in A_{630} of the CAS dye reaction mixture as reported previously (3). Siderophore activities are expressed relative to uninoculated complete culture medium. Siderophore assays were performed in triplicate in multiple experimental trials. Purified alcaligin used in control siderophore activity assays and spectroscopic analysis was isolated from *B. bronchiseptica* cultures by the simplified large-scale method described by Brickman and coworkers (10). Determination of hydroxamates was by the method of Csaky (15) with hydroxylamine hydrochloride as standard. Visible and UV spectroscopy used a Beckman DU-65 spectrophotometer (Beckman Instruments, Inc., Fullerton, Calif.).

Inhibition of alcaligin siderophore production by a competitive inhibitor of Odc activity. Wild-type *B. bronchiseptica* B013N was cultured overnight in iron-replete SS, and then bacterial cells from 200 μl of culture were harvested by centrifugation, washed twice with 1-ml volumes of iron-depleted SS, and used to seed 20 ml of iron-depleted SS. The resulting suspension was divided among sterile culture tubes, and a 0.5 M stock solution of aqueous DAB, a competitive inhibitor of Odc activity (13), was added to the first tube to provide 2 mM final concentration. DAB was diluted serially 1:5 through the series of culture tubes, yielding starting cultures containing DAB at 2.000, 0.400, 0.080, 0.016, 0.003, and 0.001 mM final concentrations. A control culture had no DAB added. Cultures were incubated with shaking at 37°C for 18 h and then assayed for siderophore production by the CAS method.

Odc enzyme assays. Odc activities of *Bordetella* strains were assayed by a modification of the method described by Tabor and coworkers (44, 45) for mass screening of polyamine biosynthetic mutants. Odc activities were determined for *Bordetella* strains grown under iron-replete or iron-depleted SS culture conditions to examine whether expression of Odc activity was iron regulated. *Bordetella* strains grown for approximately 20 h in iron-replete SS were subcultured 1:200 into iron-replete or iron-depleted SS and grown with shaking at 37°C for an additional 16 to 18 h, corresponding to mid- to late logarithmic growth phase. A volume of each culture equivalent to 2 optical density units (600-nm wavelength) of bacterial cells (usually 0.5 to 1 ml) was pelleted gently in a microcentrifuge, washed twice with 1-ml volumes of a cold buffer consisting of 20 mM Tris-HCl (pH 8.0)–5 mM dithiothreitol–1 mM EDTA (TDE buffer), and resuspended in 100 μl of cold TDE. Twofold serial dilutions of cell suspensions were prepared in cold TDE buffer, and then to each dilution an equal volume of cold TDE–2% toluene emulsion was added. Fifty-microliter volumes of the resulting cell suspensions in TDE–1% toluene, equivalent to 2×10^8, 1×10^8, and 5×10^7 bacterial cells on the basis of absorbance measurements, were plated in triplicate in 96-well trays and incubated at 37°C for 30 min for permeabilization. Fifty microliters of an Odc reaction mixture consisting of 20 mM Tris-HCl (pH 8.0)–5 mM dithiothreitol–50 μM pyridoxal phosphate–1 mg of bovine serum albumin ml^{-1}–100 μM L-[1-^{14}C]ornithine (0.3 μCi/ml) was added to each well, and then the plates were covered with filter paper wetted with a freshly prepared saturated solution of barium hydroxide to trap evolved $^{14}CO_2$ as insoluble barium carbonate. The 96-well tray lids were replaced, and the plates were wrapped in plastic wrap, clamped securely to provide tight contact of the filter paper over the wells, and incubated at 37°C. After 2 to 3 h, the filter paper was removed, rinsed briefly in acetone, air dried, and subjected to autoradiography for 2 to 5 days. Autoradiographs were optically scanned, and digitized images were integrated with the National Institutes of Health Image version 1.57 image processing and analysis program for the Macintosh computer. The areas of the dried filters corresponding to each well were excised, and captured [^{14}C]barium carbonate was quantified with EcoLume scintillant (ICN Biomedicals, Inc.) in a Beckman LS5000TD liquid scintillation counter (Beckman Instruments, Inc.). Nonenzymatic background levels of $^{14}CO_2$ evolution in Odc assays were determined with B013N cell suspensions that were boiled for 10 min prior to dilution with TDE–2% toluene. Siderophore levels of the cultures used in these experiments were monitored by CAS assay.

Construction of a *B. pertussis odc* mutant by allelic exchange. The 5′-coding region of the *B. pertussis odc* gene was deleted from a cloned 8.5-kb *Xho*I DNA fragment by substitution of an internal 3.8-kb *Eco*RI *B. pertussis* DNA fragment with a 2.1-kb Tn*5*-derived kanamycin resistance cassette in the allelic exchange plasmid vector pRTP1 (42). The resulting deletion-replacement plasmid construct was conjugally transferred to a spontaneous streptomycin-resistant derivative of *B. pertussis* UT25, UT25Sm1 (2), and the mutation was transferred to the chromosome by homologous recombination as described by Stibitz and coworkers (42). Reciprocal exchange was verified by Southern hybridization analysis. The resulting Δ*odc*::Kan *B. pertussis* mutant was designated P12.

Nucleotide sequence accession number. The GenBank accession number assigned to the partial *B. pertussis* UT25 *odc* nucleotide sequence is U34895.

RESULTS

Transposon insertions defining group III siderophore-deficient mutants of *B. bronchiseptica* interrupt a genetic region predicted to encode an amino acid decarboxylase. Molecular cloning of mini-Tn*5*/*lacZ1* transposon insertion markers associated with group III *B. bronchiseptica* siderophore-deficient mutants BRM3 and BRM5, resulting in recombinant plasmids pBRM3 and pBRM5, was accomplished in a previous study (3). *B. bronchiseptica* genomic sequences flanking the inser-

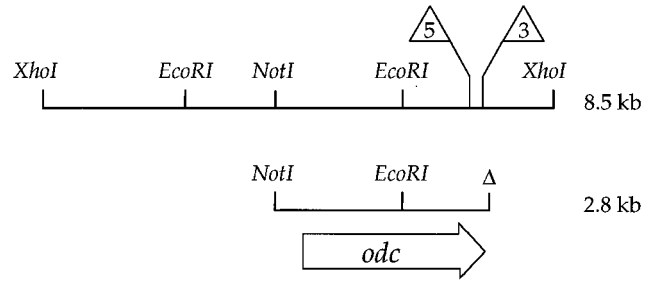

FIG. 1. Restriction map of the *B. pertussis* UT25 genomic region complementing alcaligin siderophore-deficient group III *B. bronchiseptica* mutants BRM3 and BRM5. The upper map depicts the 8.5-kb *Xho*I DNA fragment shared by four distinct group III-complementing *B. pertussis* cosmids. The relative positions of opposing mini-Tn*5*/*lacZ1* transposon insertions defining BRM3 and BRM5 are indicated by the triangles (3, BRM3 insertion, and 5, BRM5 insertion). The lower map shows the limits of the minimal complementing 2.8-kb *B. pertussis* DNA fragment identified. The Δ symbol indicates that one terminus of the fragment was generated by exonuclease III treatment. The relative position of an open reading frame resembling pyridoxal phosphate-dependent amino acid decarboxylases is indicated by the arrow labeled *odc*.

tions were used in DNA hybridization analysis to demonstrate the existence of homologous sequences in the related species *B. pertussis*. Restriction mapping estimated that the chromosomal sites of the BRM3 and BRM5 insertions were separated by approximately 200 to 300 bp. BRM3 and BRM5 displayed no significant growth defect other than the inability to cope with iron starvation.

To isolate the *B. pertussis* homolog of the mutated group III siderophore gene(s), an approximately 700-bp *Not*I-*Sal*I fragment of pBRM3 was used to probe a *B. pertussis* UT25 cosmid-based genomic library by in situ DNA hybridization. Four distinct recombinant cosmids that hybridized strongly with the *B. bronchiseptica* DNA probe at high stringency were identified. Restriction mapping and Southern hybridization analysis of the four cosmids, pCP3.1, pCP3.2, pCP3.3, and pCP3.4, determined that they shared *B. pertussis* DNA sequences homologous to the *B. bronchiseptica* DNA region interrupted by the insertions defining the group III siderophore-deficient mutants (data not shown). All four *B. pertussis* cosmids restored wild-type levels of iron-regulated siderophore production to mutants BRM3 and BRM5 when introduced by conjugal transfer, as determined by the CAS siderophore assay and absorption spectroscopy of iron-depleted culture supernatants. Subcloning and complementation analysis established that the cosmids shared an 8.5-kb *Xho*I *B. pertussis* UT25 genomic DNA fragment that encoded the complementing activity (Fig. 1). Cosmid pCP3.3 was selected for further examination. Complementation analysis of pCP3.3 plasmid subclones and deletion derivatives mapped the genetic region encoding the complementing activity to an approximately 2.8-kb *B. pertussis* DNA fragment extending from a *B. pertussis Not*I restriction site to a terminus generated by exonuclease III treatment; the genetic limits of this fragment are depicted in Fig. 1. The minimal complementing plasmid pRK/143.1 carries the 2.8-kb fragment in the broad-host-range plasmid vector pRK415.

Nucleotide sequencing analysis of pBRM3 and pBRM5 with synthetic oligonucleotide primers complementary to the mini-Tn*5*/*lacZ1* transposon "I" and "O" ends confirmed that the BRM3 and BRM5 chromosomal insertions were separated by 222 bp. Nucleotide sequences flanking the cloned transposon insertions were identical to corresponding sequences determined later for the complementing *B. pertussis* DNA. Nucleotide sequencing of pCP3.3 plasmid subclones and deletion derivatives and subsequent BLAST database searches of the translated *B. pertussis* DNA sequences identified high-scoring segment pairs with a family of pyridoxal phosphate-dependent amino acid decarboxylase protein sequences. These decarboxylases included LDC, the lysine decarboxylase of *Hafnia alvei* (17); CadA, the inducible lysine decarboxylase of *E. coli* (32); Adi, the biodegradative arginine decarboxylase of *E. coli* (43); SpeC, the constitutive biosynthetic Odc of *E. coli* (4); and SpeF, the inducible Odc of *E. coli* (25). The putative *B. pertussis* decarboxylase homolog was encoded by a DNA region corresponding to the *B. bronchiseptica* genomic region disrupted by the transposon insertions defining BRM3 and BRM5 (Fig. 1).

Over the highest-scoring 63-amino-acid segment of similarity with the decarboxylases, multiple sequence alignments found 54% identity of the deduced partial *Bordetella* amino acid sequence with LDC, 52% identity with CadA, 46% with Adi, and 38% with both SpeC and SpeF. A multiple amino acid sequence alignment of this region is shown in Fig. 2. Percent identity between the deduced *B. pertussis* polypeptide and each of the same known decarboxylases over a 20-amino-acid region spanning the highly conserved pyridoxal phosphate cofactor-binding site ranged from 50 to 80.

Expression of a putative *Bordetella* amino acid decarboxylase protein in *E. coli*. The 2.8-kb *B. pertussis* DNA fragment including the region encoding the putative pyridoxal phosphate-dependent amino acid decarboxylase homolog was subcloned into plasmid vector pGEM4Z to produce pT7/*odc*, allowing bacteriophage T7 promoter-directed expression of *Bordetella* proteins in *E. coli* K38 (pGP1-2). Plasmid pT7/*odc*-encoded proteins were exclusively ^{35}S radiolabeled and analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography. A *B. pertussis* DNA-encoded product of approximately 86 kDa in apparent molecular mass was consistently observed in autoradiographs of SDS-polyacrylamide gels (Fig. 3), as was the T7-transcribed vector β-lactamase product(s) also seen in pGEM4Z vector control samples. The observed 86-kDa molecular-mass product is within the calculated limits of the coding capacity of the 2.8-kb DNA fragment and approximates the 82- to 84-kDa mass of the homologous decarboxylases identified in database searches.

Restoration of alcaligin siderophore production to mutant BRM3 by culture medium supplementation with amino acid decarboxylase products. In an attempt to circumvent the siderophore biosynthesis defect associated with the loss of a putative amino acid decarboxylase activity in group III mutant BRM3, iron-depleted SS culture medium was supplemented with cadaverine, agmatine, or putrescine, which are the prod-

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      S G D H W H G F G D L A P G F N M L D P I K A T I I T P G L D - M  Odc
469   P R N E W H G F P N I D N D H M Y L D P I K V T L L T P G L S - P  LDC
469   S D S T W H G F K N I D N E H M Y L D P I K V T L L T P G M E - K  CadA
514   P G E S W H G F K D I P D N W S M L D P I K V S I L A P G M G - E  Adi
486   P G A K W H G F E G Y A A D Q Y F V D P C K L L L T T P G I D A E  SpeC
474   P G E H W H S F E G Y A E N Q Y F V D P C K L L L T T P G I D A R  SpeF

      S G S F G E T G I P A A L V S K Y L A E H G V V V E K - - T G L Y  Odc
501   N G T L E E E G I P A S I V S K Y L D E H G I I V E K - - T G P Y  LDC
501   D G T M S D F G I P A S I V A K Y L D E H G I V V E K - - T G P Y  CadA
546   D G E L E E T G V P A A L V T A W L G R H G I V P T R - - T T D F  Adi
519   T G E Y S D F G V P A T I L A H Y L R E N G I V P E K C D L N S I  SpeC
507   N G E Y E A F G V P A T I L A N F L R E N G V V P E K C D L N S I  SpeF
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FIG. 2. Multiple primary amino acid sequence alignment of translated *B. pertussis* DNA sequences with amino acid decarboxylases identified in BLAST database searches. The partial decarboxylase sequences shown represent the highest-scoring 63-amino-acid segment of similarity with a deduced *B. pertussis* polypeptide sequence. Residue positions of translated decarboxylase amino acid sequences are numbered as they appear in the GenBank database submissions (GenBank accession numbers: *B. pertussis* Odc, U34895; *H. alvei* LDC, P05033; *E. coli* CadA, P23892; *E. coli* Adi, P28629; *E. coli* SpeC, P21169; *E. coli* SpeF, P24169).

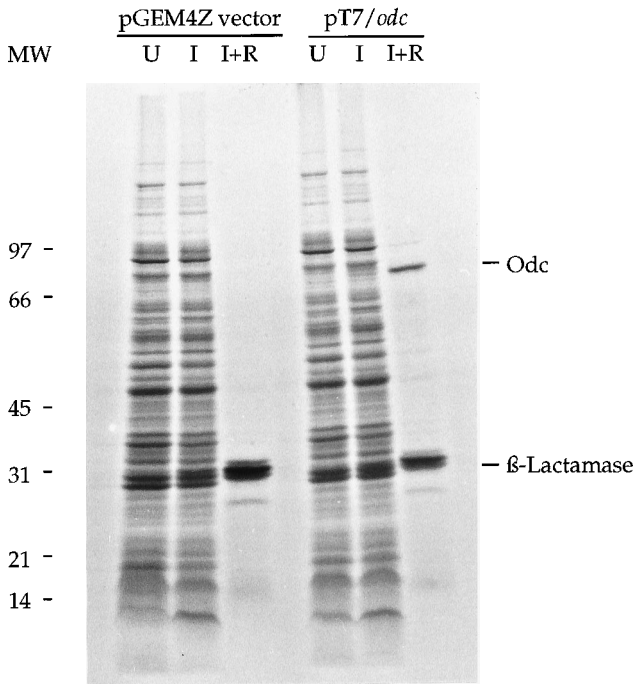

FIG. 3. Expression of an 86-kDa polypeptide encoded by the *B. pertussis odc* region with a bacteriophage T7 RNA polymerase-promoter expression system in *E. coli*. Samples: pGEM4Z, K38 (pGP1-2, pGEM4Z) vector control; pT7/*odc*, K38 (pGP1-2, pT7/*odc*). U, uninduced cultures; I, induced for expression of T7 promoter-directed genes by temperature shift; I+R, induced by temperature shift in the presence of rifampin. Positions of marker proteins are given along with their apparent molecular masses in kilodaltons. Positions of radiolabeled products corresponding to the vector-encoded β-lactamase product(s) and the *B. pertussis* DNA-encoded product Odc are indicated.

ucts of lysine decarboxylase, arginine decarboxylase, or Odc activities, respectively. Iron-regulated alcaligin production by BRM3 was restored to wild-type levels by medium supplementation with putrescine (1,4-diaminobutane) and only partially

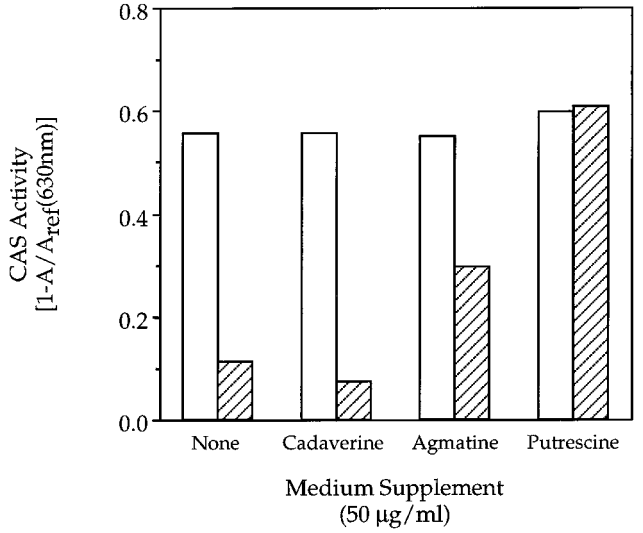

FIG. 4. Alcaligin siderophore production by *B. bronchiseptica* in culture medium supplemented with amino acid decarboxylase products. Mean relative CAS siderophore activities ($1 - A/A_{ref}$, $n = 3$) of wild-type parent strain B013N (open bars) and group III mutant BRM3 (hatched bars) after 24 h of growth in unsupplemented medium (None) or in culture medium supplemented with 50 μg of cadaverine, agmatine, or putrescine per ml are shown.

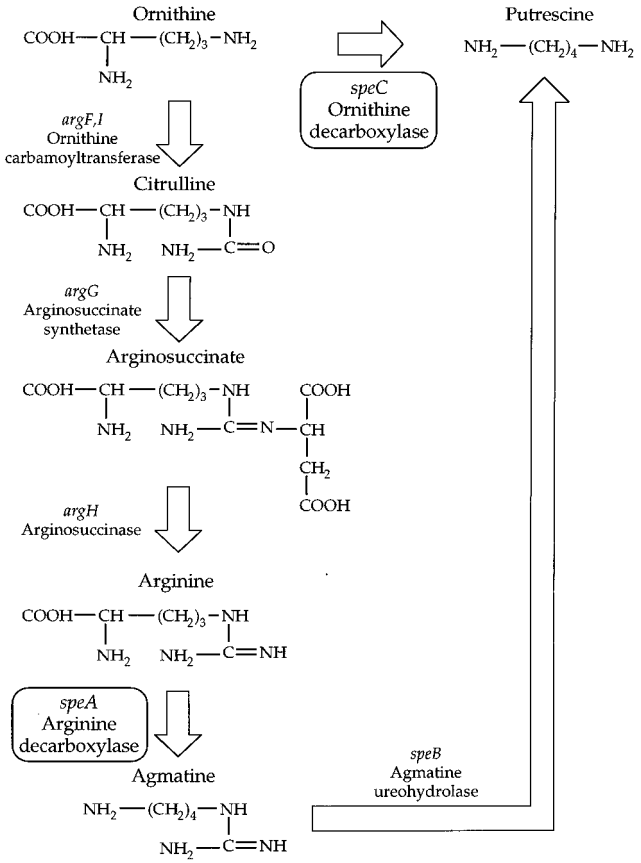

FIG. 5. Alternative pathways for biosynthesis of putrescine in *E. coli* involve SpeA (arginine decarboxylase) or SpeC (Odc).

restored by supplementation with agmatine (1-amino-4-guanidobutane) (Fig. 4). Cadaverine supplementation had no effect on siderophore production by BRM3, nor did supplementation with ornithine, arginine, or spermidine in other experiments. All supplements had no effect on alcaligin production by wild-type B013N, except putrescine, which resulted in a slight enhancement of siderophore production.

In *E. coli*, putrescine can be produced by two alternative biochemical pathways (34): the first and most efficient route is direct decarboxylation of ornithine, and the second is a two-step process involving decarboxylation of arginine to form agmatine, followed by the conversion of agmatine to putrescine by the action of agmatine ureohydrolase (Fig. 5). Full restoration of siderophore production to BRM3 by putrescine and partial restoration by agmatine were consistent with the predicted involvement of an amino acid decarboxylase activity, possibly either an Odc or an arginine decarboxylase, in the production of putrescine, the proposed precursor of the *Bordetella* siderophore alcaligin on the basis of structural analysis.

Inhibition of *Bordetella* alcaligin production by an inhibitor of Odc. Supplementation of iron-depleted SS culture medium with the Odc inhibitor DAB resulted in strong reduction in siderophore levels achieved by wild-type *B. bronchiseptica* B013N (Fig. 6). Alcaligin production was highly sensitive to DAB inhibition; the lowest inhibitor concentration tested, 0.001 mM, decreased siderophore production by approximately 20%. At 0.080 mM DAB, inhibition was near maximal at approximately 50% inhibition of siderophore production compared with untreated cultures. DAB concentrations used

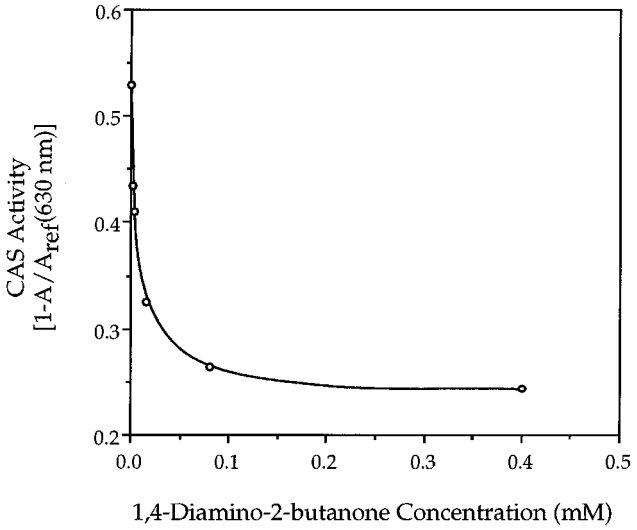

FIG. 6. Inhibition of alcaligin siderophore production in *B. bronchiseptica* by an inhibitor of Odc. Mean relative CAS siderophore activities ($1 - A/A_{ref}$, $n = 3$) of 18-h B013N cultures are plotted as a function of concentration (millimolars) of the Odc inhibitor DAB added to the culture medium.

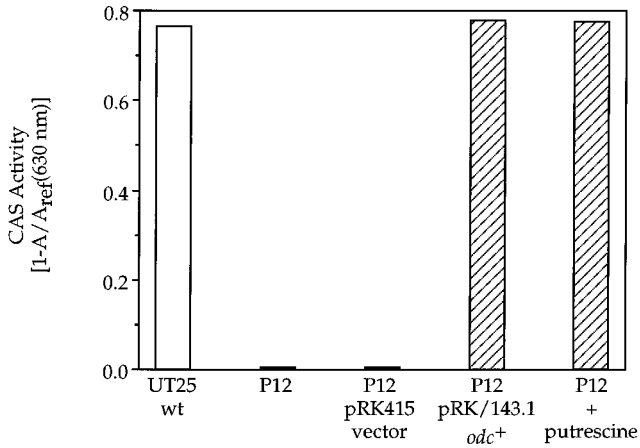

FIG. 7. Alcaligin siderophore production by *B. pertussis odc* mutant P12 constructed by allelic exchange. Mean relative CAS siderophore activities ($1 - A/A_{ref}$, $n = 3$) of 24-h cultures of *odc* mutant P12 carrying the designated plasmids or supplemented with putrescine are shown versus that of the wild-type parent strain UT25. wt, wild type.

were not growth inhibitory over the culture period examined, and control assays established that DAB did not interfere with the CAS siderophore assay.

Restoration of siderophore production to mutant BRM3 by the *speC* constitutive Odc-encoding gene of *E. coli*. Concordant with the decarboxylase product feeding results, the siderophore biosynthesis defect of BRM3 was functionally complemented in *trans* by plasmid pRK/3.2P, encoding the heterologous Odc activity SpeC of *E. coli* (8) (Table 1). Alcaligin levels produced by BRM3 (pRK/3.2P) approximated those of wild-type *B. bronchiseptica* B013N and BRM3 carrying the group III-complementing *B. pertussis* plasmid pRK/143.1.

Restoration of Odc activity to BRM3 by plasmids complementing its siderophore biosynthesis defect. In Odc assays, group III siderophore-deficient mutant BRM3 was found to lack significant levels of Odc activity compared with the wild-type parent strain B013N (Table 1). Counts determined were in close agreement with integrated values resulting from computer analysis of digitized autoradiographic images. Activities for mutant BRM3 and BRM3 carrying the plasmid vector pRK415 were essentially the same as nonenzymatic background activity of boiled B013N suspensions. Levels of Odc activity comparable to those expressed by wild-type B013N were restored to BRM3 by the *B. pertussis* plasmid, pRK/143.1, that complemented its siderophore biosynthesis defect, indicating that the amino acid decarboxylase activity required for alcaligin biosynthesis is an Odc. *Bordetella* Odc enzymatic activities appeared to be largely independent of iron concentration in the culture medium, suggesting that the previously reported Fur-mediated iron regulation of *Bordetella* siderophore biosynthesis (9) is exerted over other alcaligin biosynthetic activities.

TABLE 1. Alcaligin siderophore production and Odc activities of *B. bronchiseptica*

Strain	Alcaligin siderophore production[a]		Odc activity[b]	
	+ Iron[c]	− Iron[d]	+ Iron	− Iron
B013N (*wt*)	0.019	0.574	362	407
BRM3	0.014	0.052	46	34
BRM3 (pRK415 vector)	0.012	0.085	26	7
BRM3 (pRK143.1) *odc*$^+$	0.013	0.570	338	363
BRM3 (pRK3.2P) *speC*$^+$	0.013	0.543	ND[e]	ND

[a] Mean relative CAS siderophore activities ($1 - A/A_{ref}$, $n = 3$) of 24-h SS cultures.
[b] Mean disintegrations per minute ($^{14}CO_2$, $n = 3$) evolved over a 2-h reaction period by 2×10^8 bacterial cells.
[c] + Iron, iron-replete culture conditions.
[d] − Iron, iron-depleted culture conditions.
[e] ND, not determined.

Construction and analysis of a group III-analogous mutant of *B. pertussis*. Siderophore-deficient *B. pertussis* mutant P12 was constructed by allelic exchange by using a deletion-replacement pRTP1 derivative to delete approximately 1 kb of N-terminal coding sequences of the *B. pertussis* Odc-encoding gene required for alcaligin biosynthesis. Deletion of the 3.8-kb *B. pertussis* genomic DNA fragment depicted in Fig. 1 and replacement by the 2.1-kb Tn*5*-derived kanamycin resistance cassette were confirmed by Southern hybridization analysis of genomic DNA isolated from a dozen independent kanamycin-resistant derivatives. As with *B. bronchiseptica* mutant BRM3, *B. pertussis* P12 was incapable of alcaligin siderophore production unless supplied with the wild-type DNA sequences in *trans* or unless the iron-depleted growth medium was supplemented with putrescine (Fig. 7). These data establish that the Odc, for which we propose the genetic designation *odc*, is required for biosynthesis of the siderophore alcaligin (Fig. 8) in *B. pertussis* as well as in *B. bronchiseptica*.

DISCUSSION

We report the identification of the *odc* gene encoding an enzymatic activity essential for alcaligin siderophore biosynthesis in *Bordetella* spp. This Odc supplies the alcaligin precursor 1,4-diaminobutane, commonly known as putrescine. The involvement of an amino acid decarboxylase activity in the biosynthesis of alcaligin was predicted on the basis of database searches of translated DNA sequences corresponding to the genomic region interrupted by transposon insertions defining previously reported *B. bronchiseptica* alcaligin-deficient mu-

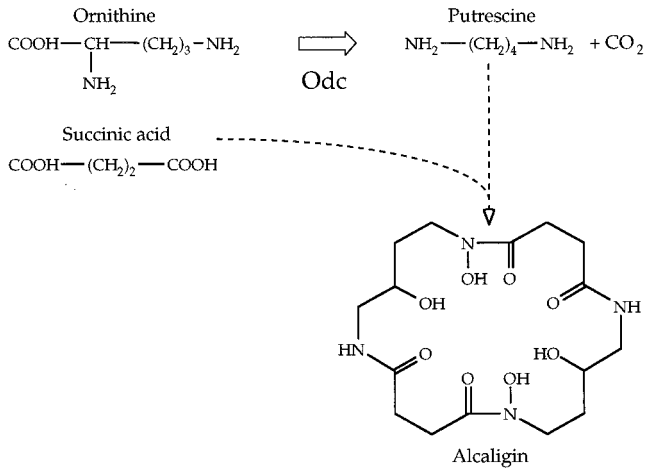

FIG. 8. Role of Odc in the biosynthetic pathway for *Bordetella* alcaligin. The *odc* gene encodes an Odc activity essential for production of the alcaligin precursor putrescine. Dashed arrows indicate unknown biosynthetic activities.

tants BRM3 and BRM5. Evidence supporting the involvement of an Odc activity was gained in mutant feeding experiments in which culture medium supplementation with decarboxylated ornithine (putrescine) fully restored alcaligin biosynthetic capacity to BRM3. Accordingly, the alcaligin biosynthesis defect of BRM3 was functionally complemented by expression of a heterologous Odc activity, SpeC of *E. coli*. Ultimately, BRM3 was shown to be defective in Odc activity compared with its wild-type parent B013N in crude enzyme assays, and wild-type Odc activity levels were restored to BRM3 by plasmids shown to complement its alcaligin biosynthesis defect. An Odc mutant of *B. pertussis* constructed by allelic exchange was likewise unable to produce alcaligin. The identification of putrescine as a precursor of alcaligin in *Bordetella* spp. is in agreement with earlier predictions based on the known structure of the siderophore.

The siderophore alcaligin was first purified from the taxonomically related species *A. denitrificans* subsp. *xylosoxydans*, isolated from freshwater lake sediment (35, 36). Structural analysis found alcaligin to be a symmetric 20-membered ring, 1,8(*S*),11,18(*S*)-tetrahydroxy-1,6,11,16-tetraazacycloeicosane-2,5,12,15-tetrone. The dihydroxamate molecule can thus be considered a cyclic dimer of two structural units consisting of 1-amino-4-(*N*-hydroxylamino)-2(*S*)-butanol and succinic acid. Consistent with this twofold symmetric structure, high-resolution proton and carbon nuclear magnetic resonance spectroscopy recently determined that the molecule displays a twofold symmetric conformation in aqueous solution, although evidence suggests that multiple C_2-symmetric conformations of alcaligin coexist in aqueous and methanolic solution (10). Alcaligin shares structural features with the ferrioxamine family of siderophores (30), which are produced by actinomycetes as well as certain enterobacterial species such as *H. alvei* (55). All ferrioxamines incorporate hydroxylated polyamines and succinic acid in their molecular structures. It is possible that LDC of *H. alvei* identified in database searches as a homolog of *Bordetella* Odc may be required for production of the cadaverine portion of *Hafnia* ferrioxamines. Alcaligin most resembles the only other known cyclic dihydroxamate siderophore, bisucaberin, produced by the marine bacterium *Alteromonas haloplanktis* (23, 48). However, bisucaberin includes two residues of *N*-hydroxycadaverine instead of the hydroxyl-substituted *N*-hydroxyputrescine residues of alcaligin.

Certain catechol-type siderophores, such as agrobactin (37), vibriobactin (20), and parabactin (47) also contain polyamines, and in *Paracococcus denitrificans*, along with parabactin siderophore production, spermine production increases by an order of magnitude in response to iron starvation, although spermidine and not spermine donates its triamine backbone to the siderophore (5).

Remarkable parallels exist between the *Bordetella odc* gene and the recently described *hdc* gene of *Vibrio anguillarum* required for biosynthesis of the siderophore anguibactin (49), a trait necessary for virulence. As with *Bordetella odc*, the *V. anguillarum hdc* gene was identified after transposon mutagenesis on the basis of a siderophore-deficient phenotype. Nucleotide sequence analysis of the mutated region of plasmid pJM1 identified deduced amino acid sequence similarities with known histidine decarboxylases. Anguibactin siderophore production by *V. anguillarum hdc* mutants was restored by culture medium supplementation with histamine, the product of histidine decarboxylation, confirming the predicted role of histamine as an essential precursor of that siderophore. Further, neither *odc* nor *hdc* is repressible by iron, although alcaligin and anguibactin siderophore production have been shown to be negatively regulated by the action of a Fur repressor (9, 50), indicating that iron concentration controls other siderophore biosynthetic activities.

On the basis of the elucidation of the alcaligin biosynthesis role of *odc* in *Bordetella* spp. and the known structure of alcaligin, we hypothesize the subsequent action of an oxygenase catalyzing the hydroxylation of putrescine. This putative *Bordetella* oxygenase would supply an activity analogous to the IucD L-lysine N^6-hydroxylase of *E. coli* (22), involved in the biosynthesis of the hydroxamate siderophore aerobactin, and to the L-ornithine N^5-oxygenases PvdA of *Pseudomonas aeruginosa* (51) and Sid1 of *Ustilago maydis* (31), required for pyoverdin and ferrichrome siderophore biosynthesis, respectively. Preliminary nucleotide sequencing of the *B. pertussis* DNA corresponding to the *B. bronchiseptica* genomic region interrupted by transposons defining the group I alcaligin siderophore-deficient mutants BRM1 and BRM9 (GenBank accession number U34894) identified an open reading frame predicted to encode such a *Bordetella* homolog of the IucD, PvdA, and Sid1 oxygenases (24). Confirmation of the role of this putative *Bordetella* oxygenase in the biosynthesis of alcaligin awaits further biochemical analysis.

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