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Identification of a Plasmid-Encoded Gene from *Haemophilus ducreyi* Which Confers NAD Independence

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Members of the family *Pasteurellaceae* are classified in part by whether or not they require an NAD supplement for growth on laboratory media. In this study, we demonstrate that this phenotype can be determined by a single gene, *nadV*, whose presence allows NAD-independent growth of *Haemophilus influenzae* and *Actinobacillus pleuropneumoniae*. This gene was cloned from a 5.2-kb plasmid which was previously shown to be responsible for NAD independence in *Haemophilus ducreyi*. When transformed into *A. pleuropneumoniae*, this cloned gene allowed NAD-independent growth on complex media and allowed the utilization of nicotinamide in place of NAD on defined media. Sequence analysis revealed an open reading frame of 1,482 bp that is predicted to encode a protein with a molecular mass of 55,619 Da. Compared with the sequence databases, NadV was found to have significant sequence homology to the human pre-B-cell colony-enhancing factor PBEF and to predicted proteins of unknown function identified in the bacterial species *Mycoplasma genitalium, Mycoplasma pneumoniae, Shewanella putrefaciens, Synechocystis sp., Deinococcus radiodurans, Pasteurella multocida*, and *Actinobacillus actinomycetemcomitans*. *P. multocida* and *A. actinomycetemcomitans* are among the NAD-independent members of the *Pasteurellaceae*. Homologues of NadV were not found in the sequenced genome of *H. influenzae*, an NAD-dependent member of the *Pasteurellaceae*, or in species known to utilize a different pathway for synthesis of NAD, such as *Escherichia coli*. Sequence alignment of these nine homologues revealed regions and residues of complete conservation that may be directly involved in the enzymatic activity. Identification of a function for this gene in the *Pasteurellaceae* should help to elucidate the role of its homologues in other species.

NAD is a critical cofactor required for energy metabolism and many oxidation-reduction reactions in both prokaryotic and eukaryotic cells. In many bacterial species, synthesis of NAD occurs de novo via quinolinic acid (3, 4). NAD can also be synthesized by a pyridine nucleotide salvage pathway via nicotinic acid (3, 4). Members of the family *Pasteurellaceae* do not possess either of these pathways for NAD biosynthesis. These bacterial species must acquire this essential nutrient from their environment either as NAD directly or from a limited number of precursors (18, 19). This pyridine nucleotide requirement has been historically important in the identification and classification of members of the *Pasteurellaceae*, with species requiring an NAD supplement for growth in vitro described as V-factor dependent (12, 13). In V-factor-dependent species, the pyridine nucleotide source must possess an intact pyridine-ribose bond and the pyridine-carbonyl group must be amidated; therefore, nicotinamide mononucleotide (NMN) and nicotinamide riboside (NR) can function as V-factors, but quinolinic acid, nicotinic acid, nicotinic acid mononucleotide, and nicotinamide (NAm) cannot (3, 19). The ability to use NAm as a precursor for NAD biosynthesis has been shown to differentiate V-factor-dependent from V-factor-independent strains among the *Pasteurellaceae* (20). *Haemophilus haemoglobinophilus*, which is V-factor independent, synthesizes the enzyme nicotinamide phosphoribosyltransferase, which converts NAm to NMN and allows the use of NAm as a source of pyridine nucleotide (11) (Fig. 1). Since NAm is available in most complex bacteriologic media, bacteria that can utilize NAm are V-factor independent.

For many species of *Pasteurellaceae* defined as V-factor dependent, V-factor-independent variants have been identified. These include strains of *Actinobacillus pleuropneumoniae*, which causes pleuropneumonia in swine (22); *Haemophilus paragallinarum*, which causes fowl choryza (1, 15); *Haemophilus parainfluenzae*, which can cause pneumonia and meningitis in humans (7); and *Haemophilus ducreyi*, which causes the sexually transmitted disease chancroid in humans (26, 27). In *H. parainfluenzae, H. paragallinarum*, and *H. ducreyi*, V-factor independence has been shown to be encoded on a plasmid (1, 27, 28). However, in *A. pleuropneumoniae*, it appears that V-factor independence may be conferred by a chromosomal gene (10).

In this paper, we report the cloning and sequence analysis of a plasmid-encoded gene in a V-factor-independent strain of *H. ducreyi*. This gene was capable of conferring NAD independence to a variety of *Pasteurellaceae* and appears to be widely distributed among both prokaryotic and eukaryotic organisms.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *Escherichia coli* XL1-Blue MRF′ (Stratagene, La Jolla, Calif.) was used for propagation of the plasmid vectors pUC18 (Gibco-BRL, Rockville, Md.) and pGZRS18 (25) as well as derivatives of these plasmids. *E. coli* strains were grown on Luria-Bertani (LB) medium supplemented with ampicillin (100 μg/ml) for plasmid selection. *A. pleuropneumoniae* ATCC 27088 and *H. influenzae* KW20 Rd- (2) strains were grown at 37°C under a 5% CO_2 atmosphere on brain heart infusion (BHI) broth or agar (Difco Laboratories, Detroit, Mich.) supplemented with V-factor (NAD) and X-factor (hemin), both at 10 μg/ml, and ampicillin at 50 μg/ml as needed. NAD was

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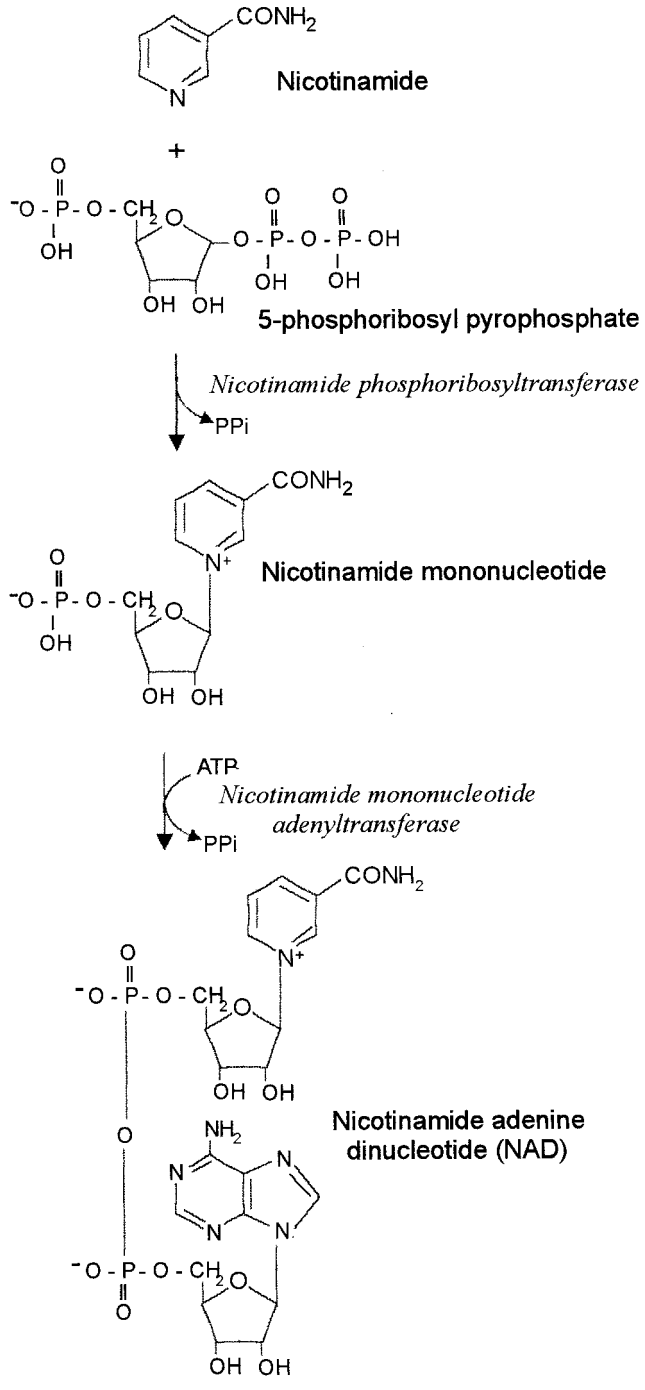

FIG. 1. Biochemical pathway for the biosynthesis of NAD as found in the family *Pasteurellaceae*. NAD-dependent species lack the ability to convert NAm to NMN.

omitted when selecting for V-factor-independent transformants. *H. ducreyi* ATCC 27722 was grown on chocolate agar (BHI agar base plus 5% boiled sheep blood plus 1% IsoVitaLex) at 35°C in a candle jar.

The defined medium used to grow *A. pleuropneumoniae* and *H. influenzae* was a modification of the recipe developed by Herriott for *H. influenzae* (9), with 10 mM glucose added and the amino acid stock solution from the *Neisseria* defined medium developed by Morse and Bartenstein (16) substituted for Herriott's amino acid solution. This medium was supplemented with hemin (10 μg/ml) and with 10 μg/ml NAD or NAm (Sigma Chemical Co., St. Louis, Mo.) as needed to determine specific nutritional requirements.

DNA manipulations. Restriction enzymes, calf intestinal phosphatase, and DNA ligase were purchased from Boehringer Mannheim Biochemicals (Indianapolis, Ind.) and used according to the manufacturer's instructions. DNA fragments for subcloning were purified from agarose gels by excising the bands and isolating the DNA with Qiaex beads (Qiagen Inc., Valencia, Calif.). Plasmid DNA was isolated from *E. coli, A. pleuropneumoniae, H. ducreyi*, and *H. influenzae* using the QIAprep spin plasmid purification kit (Qiagen). *E. coli* was transformed with plasmids using the method of Hanahan (8). Plasmids were transformed into *H. influenzae* using methods described by Herriott (9). Plasmids were introduced into *A. pleuropneumoniae* by electroporation as previously described (5).

DNA sequencing. Templates for DNA sequence analysis were constructed by subcloning fragments generated from defined restriction sites within pNAD1 into pUC18. Remaining gaps in the sequence were filled using synthetic oligonucleotide primers made at the Macromolecular Structural Facility at Michigan State University as primers for sequencing. DNA sequencing was performed using an ABI100 model 377 automated sequencer (Applied Biosystems, Foster City, Calif.). Sequence analysis was performed using the Web-based Genetics Computer Group package of programs (6). Database searches were performed using the Blast program provided by the National Center for Biotechnology Information (NCBI). Partially sequenced genomes were accessed and searched either from the NCBI genome database or from individual databases listed in and linked to the Institute for Genome Research website at http://www.tigr.org.

PCR product subcloning. The open reading frame (ORF) predicted to encode the *nadV* gene was amplified using synthetic primers MM199 (5′-GCC TGC AGA AAA ATC TTT TGA ATT ATA TAA ACA AC-3′) and MM191 (5′-GCG TAT TAA CTG CAG ATA TCA TAG CGT AGT GCG-3′), which were designed to introduce unique *Pst*I sites at either end of the ORF. The amplification product was digested with *Pst*I and ligated into pUC18 to form pCNAD9. The insert was then cloned into pGZRS18 in both forward and reverse directions to form pGZNAD9 and pGZNAD10, respectively.

Enzyme assay. The assay for synthesis of NAD from NAm was adapted from that of Kasarov and Moat (11). *A. pleuropneumoniae* serotype 1A containing either pGZNAD9 or pGZRS18 was grown overnight at 37°C in BHI broth containing NAD (10 μg/ml) and ampicillin (50 μg/ml). Cells were harvested by centrifugation, washed in sterile 0.9% saline, suspended in 0.1% of the original culture volume, and disrupted by sonication on ice. Cell debris was pelleted by centrifugation. Cell-free supernatants were combined in a reaction mix that contained 1 ml of supernatant sample, 80 mM potassium phosphate buffer (pH 7.4), 16 mM $MgCl_2$, 1 mM ATP, 5 mM phosphoribosyl pyrophosphate (PRPP; Sigma), and 2 mM NAm, and the mix was incubated at 37°C in a waterbath shaker. At designated time points, 250-μl aliquots were removed and combined with 250 μl of saline and 500 μl of methanol to stop the reaction.

Analysis of products was performed by high-pressure liquid chromatography (HPLC) using a Hewlett-Packard model 1050 system with an Alltech LiChrosorb RP-18 column (10-μm particle size, 250 by 4 mm) equipped with a guard column (LiChrosorb RP-18; 5-μm particle size; EM Separations, Wakefield, R.I.). The mobile phase consisted of two elements, with an elution gradient as described in reference 14. Eluant A was 8 mM tetrabutylammonium bromide (HPLC grade; Sigma) in 0.1 M KH_2PO_4 (pH 6.0). Eluant B was 70% eluant A and 30% methanol. Absorbance was measured at 254 nm.

In assays containing radioactive substrate, assay conditions were identical except that 350 μM carbonyl-[^{14}C]NAm (American Radiolabeled Chemicals, Inc., St. Louis, Mo.) was added in place of the 2 mM NAm. To assay for radioactive incorporation, column fractions were collected into 10 ml of Safety Solve scintillation cocktail, and radioactivity was counted on a Beckman LS 6500 scintillation counter.

Nucleotide sequence accession number. The sequences reported in this paper have been submitted to GenBank and given accession number AF273842.

RESULTS

Isolation of the NAD independence plasmid from *H. ducreyi*. *H. ducreyi* ATCC 27722 had previously been shown to contain a 5.25-kb plasmid which possessed the ability to confer NAD independence on *H. influenzae* (27). We corroborated this finding by purifying the plasmid DNA from *H. ducreyi* 27722, using this DNA to transform an NAD-dependent strain of *H. influenzae*, and selecting transformants able to grow on com-

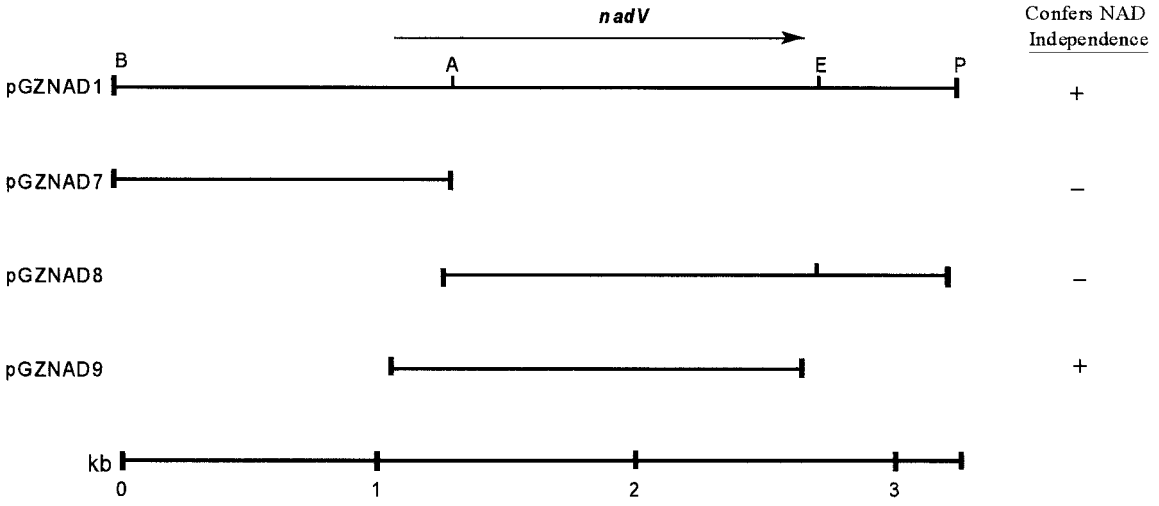

FIG. 2. Subclones of pNAD1 constructed in the *E. coli–A. pleuropneumoniae* shuttle vector pGZRS18 (25). The location of the *nadV* gene is indicated with an arrow. Plasmids pGZNAD1, pGZNAD7, and pGZNAD8 were constructed using the restriction sites shown. Plasmid pGZNAD9 was constructed using synthetic primers to PCR amplify the *nadV* gene. The ability of these clones to confer NAD independence on *A. pleuropneumoniae* is indicated in the right-hand column. Restriction sites: A, *Ava*I; B, *Bam*HI; E, *Eco*RI; and P, *Pst*I.

plex media in the absence of NAD. One of the NAD-independent colonies recovered was selected, and its plasmid content was analyzed. This transformant contained a single plasmid of approximately 5.2 kb. This plasmid was used to retransform *H. influenzae*, and NAD-independent colonies which carried the 5.2-kb plasmid were again recovered, confirming that the NAD independence phenotype was conferred by this plasmid. Thus, the *H. ducreyi* plasmid was designated pNAD1.

Localization of the NAD independence locus on pNAD1. Plasmid pNAD1 was digested with a variety of restriction enzymes, and an initial restriction map of this plasmid was used to direct the subcloning of fragments of pNAD1 into the cloning vector pUC18. The largest, a 3.3-kb *Bam*HI-*Pst*I fragment, was subcloned into the shuttle vector pGZRS18 to determine whether it contained the NAD independence locus. This subclone, pGZNAD1, was electroporated into *A. pleuropneumoniae*, and transformants were plated onto BHI agar lacking NAD. Six of the colonies recovered were found to contain a plasmid with a restriction pattern identical to that of pGZNAD1. This revealed that the gene for NAD independence was functional in *A. pleuropneumoniae* and was located on the 3.3-kb *Bam*HI-*Pst*I fragment of pNAD1 (Fig. 2).

Sequence analysis of pNAD1. The complete insert of pGZNAD1 was sequenced. The insert was 3,307 bp in length and had a G+C content of 34%. This high A+T content resulted in a high frequency of stop codons in all three reading frames. One large ORF of 1,482 bp in length was predicted to encode a protein of 494 amino acids with a molecular mass of 55,619 Da. There was an *Ava*I site located 230 bp into the ORF. Deletions made from this site in pGZNAD1 resulted in the loss of ability to complement the NAD dependence of *A. pleuropneumoniae* (Fig. 2). Based on this genetic evidence linking this ORF to the ability to confer V-factor independence on *A. pleuropneumoniae* and *H. influenzae*, the gene encoding this ORF was designated *nadV*.

To confirm that the NadV ORF conferred NAD independence, synthetic primers were used to PCR amplify the region containing the ORF and 75 bp upstream of the start codon, and the PCR product was cloned into pGZRS18 in both orientations to form pGZNAD9 (Fig. 2) and pGZNAD10. Plasmid pGZNAD9 conferred NAD independence on *A. pleuropneumoniae* but pGZNAD10 did not, suggesting that the *nadV* gene was expressed from a promoter in the vector rather than from its native promoter.

The complete nucleotide sequence and predicted amino acid sequence of *nadV* have been submitted to GenBank and given accession number AF273842. A putative ribosome-binding site was found upstream of the start codon of *nadV*. No significant inverted repeat sequences characteristic of transcriptional terminators were found downstream of the stop codon of this gene.

The predicted amino acid sequence of NadV was analyzed for the presence of functionally conserved motifs. This protein did not contain a hydrophobic, N-terminal leader sequence characteristic of secreted proteins, nor did it contain any long stretches of internal hydrophobic residues which could serve as membrane anchors. Compared against a protein motif database (6), no significant matches were found to conserved regions of previously identified protein families.

Homologues of the *nadV* gene in other organisms. The NadV sequence was used to search sequence databases. This identified one protein with a putative function and seven matches to proteins of unknown function from partially or completely sequenced microbial genomes. The protein with a putative function was the human pre-B-cell colony-enhancing factor (PBEF) protein (24). The homologues discovered in the bacterial genome databases were found in a diverse array of species, including the cyanobacterium *Synechocystis*; the radiation-resistant organism *Deinococcus radiodurans*; two *Mycoplasma* species, *M. genitalium* and *M. pneumoniae*; the gram-negative aquatic and soil organism *Shewanella putrefaciens*; and two NAD-independent members of the *Pasteurellaceae, Pasteurella multocida* and *Actinobacillus actinomycetemcomitans*. Pairwise comparisons of these sequences revealed that

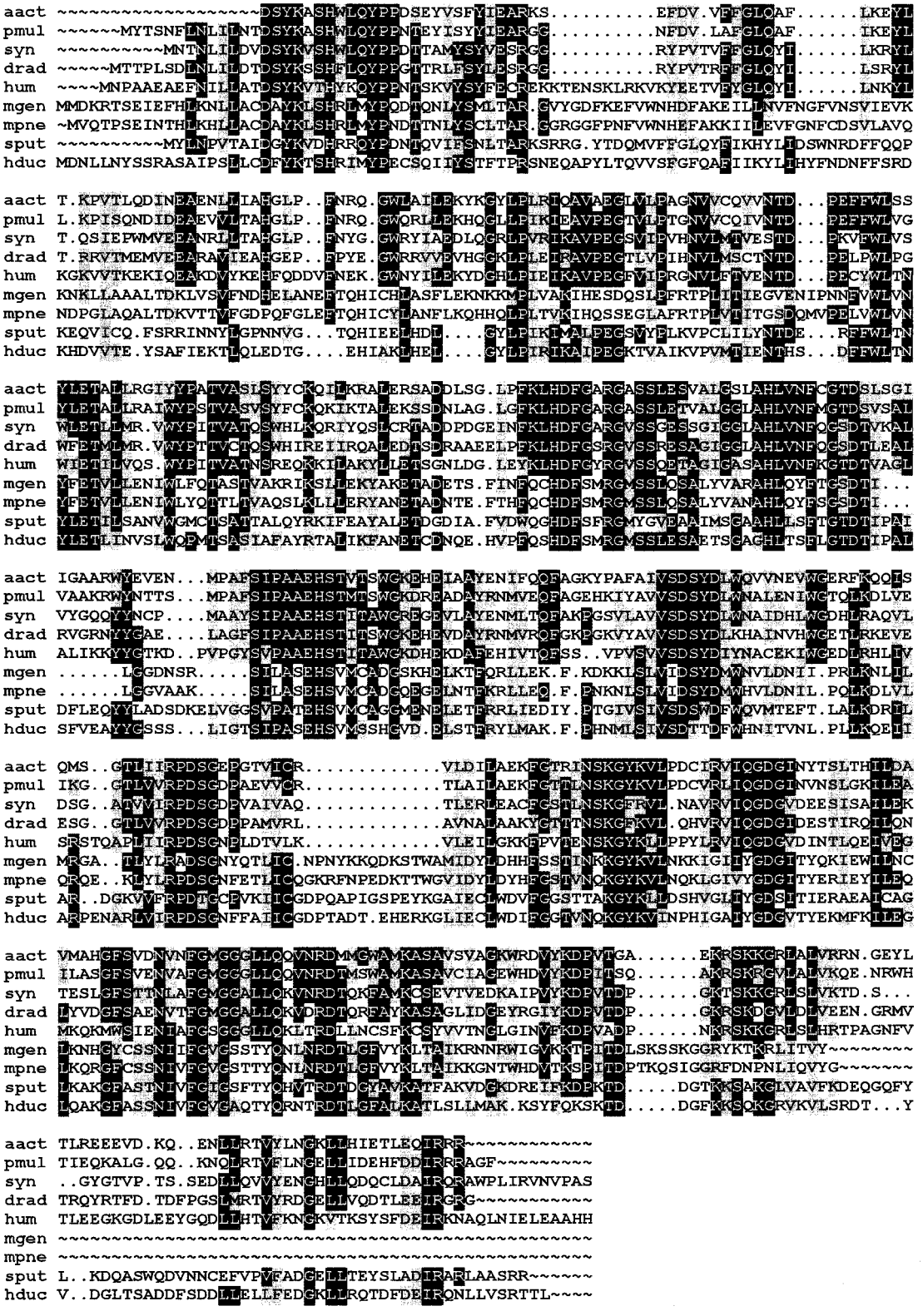

FIG. 3. Alignment of the predicted NadV amino acid sequence with homologues found in other species. Black shaded regions indicate residues that are identical in the majority of species. Gray shaded regions indicate residues that are functionally conserved in the majority of species. Species abbreviations: aact, *Actinobacillus actinomycetemcomitans*; pmul, *Pasteurella multocida*; syn, *Synechocystis*; drad, *Deinococcus radiodurans*; mgen, *Mycoplasma genitalium*; mpne, *M. pneumoniae*; sput, *Shewanella putrefaciens*; hduc, *Haemophilus ducreyi*; hum, human PBEF. The alignment was obtained using the Pileup program from the Genetics Computer Group package (6).

TABLE 1. Synthesis of NAD from NAm and PRPP by extracts of *A. pleuropneumoniae* containing *nadV*[a]

Time (min)	NAm (μmol)	NMN (μmol)	NAD (μmol)
0	360	25	25
30	220	40	123

[a] Reaction mixture contained cell extract, 80 mM potassium phosphate buffer (pH 7.4), 16 mM $MgCl_2$, 1 mM ATP, 5mM PRPP, and 2 mM NAm, incubated for 30 min at 37°C. Data are from a representative experiment. Trends were identical in all experiments.

NadV had the highest similarity to the homologue from *S. putrefaciens* and that these two were more closely related to the *Mycoplasma* homologues than to the remaining sequences. All nine sequences were aligned (Fig. 3), and numerous regions were found which contained clusters of highly conserved amino acid residues. Also conspicuous were regions where the sequences or sequence gaps from *A. actinomycetemcomitans, P. multocida, D. radiodurans, Synechocystis* sp., and human PBEF were identical but different from sequences from *M. genitalium, M. pneumoniae, S. putrefaciens*, and *H. ducreyi* NadV. This clustering is indicative of two broad families among the homologues of NadV.

Functional analysis of the NAD independence locus. Previous studies have shown that NAD-independent members of the family *Pasteurellaceae* differ from the NAD-dependent members solely in their ability to utilize the NAD precursor NAm as V-factor (18, 20). To determine whether *nadV* was responsible for this difference, *A. pleuropneumoniae* strains containing pGZNAD1, pGZNAD9, or the pGZRS18 vector were plated onto defined media lacking V-factor and onto defined media containing either NAD or NAm. All three strains failed to grow in the absence of supplement and grew in the presence of NAD, but only the strains containing the cloned *nadV* gene could grow in the presence of NAm. This indicated that the presence of the *nadV* gene allowed *A. pleuropneumoniae* to utilize NAm as a precursor for NAD biosynthesis, as diagrammed in Fig. 1, and suggests that the enzyme encoded by this gene is a novel NAm phosphoribosyltransferase (NAm-PRTase).

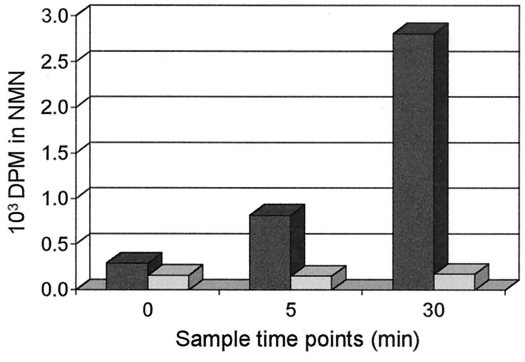

FIG. 4. Incorporation of [^{14}C]NAm into NMN. NAm-PRTase assays were performed with [^{14}C]NAm as the substrate, and incorporation of radiolabel into NMN monitored over time. Dark bars, *A. pleuropneumoniae*/pGZNAD9; light bars, *A. pleuropneumoniae*/pGZRS18.

Assay for NAm-PRTase activity. Crude cell extracts were prepared from *A. pleuropneumoniae* strains containing either pGZNAD9 or the pGZRS18 vector and assayed for the ability to synthesize NMN and NAD from NAm plus PRPP. As shown in Table 1, NAm-PRTase assays performed with extracts of *A. pleuropneumoniae* containing pGZNAD9 showed a decrease in NAm and a concomitant increase in NAD as well as a slight but consistent increase in the levels of NMN. *A. pleuropneumoniae* containing the pGZRS18 vector alone did not show an equivalent increase in NAD or decrease in NAm, nor was this pattern seen when assays with *A. pleuropneumoniae* containing pGZNAD9 were performed without PRPP in the reaction mix.

To confirm that NMN is indeed an intermediate in the biosynthesis of NAD from NAm as catalyzed by the NadV gene product, [^{14}C] NAm was used as the substrate in the same assay system. As shown in Fig. 4, ^{14}C label is incorporated into NMN by cell extracts from *A. pleuropneumoniae* containing pGZNAD9 but not in control reactions with extracts from *A. pleuropneumoniae* containing pGZRS18.

DISCUSSION

In this paper, we describe the cloning, sequence analysis, and characterization of a plasmid-encoded gene, *nadV*, from *H. ducreyi* which confers V-factor independence on several species of V-factor-dependent *Pasteurellaceae*. A 5.25-kb plasmid from *H. ducreyi* 27722 was previously described by Windsor et al. (26) and shown to confer V-factor independence on *H. influenzae* and *H. parainfluenzae*. Similar plasmids have been described in V-factor-independent strains of *H. parainfluenzae* (28) and *H. paragallinarum* (1). We isolated plasmid DNA from *H. ducreyi* 27722 and confirmed that a 5.2-kb plasmid could confer NAD independence on *H. influenzae*. We further demonstrated that subclones constructed from this plasmid in an appropriate shuttle vector could confer NAD independence on a different member of the family *Pasteurellaceae, A. pleuropneumoniae*, and that a single gene on this plasmid, *nadV*, was responsible for this phenotype.

Members of the family *Pasteurellaceae* are incapable of either de novo synthesis of NAD via quinolinic acid or recycling pyridine nucleotides via nicotinic acid (3, 4, 18), which leads to their requirement for an exogenous source of pyridine nucleotide, or V-factor. V-factor dependence in the *Pasteurellaceae* has been defined as the requirement for either NAD, NMN, or NR for growth on complex media (18). Using this definition, species such as *H. influenzae, H. parainfluenzae, H. parasuis*, and *A. pleuropneumoniae* are V-factor dependent, while *P. multocida, P. haemolytica, H. haemoglobulinophilus*, and *A. actinomycetemcomitans* are not. However, all of the members of the *Pasteurellaceae* require a pyridine nucleotide when grown on chemically defined media (18). In this case, the difference is that the V-factor-independent strains can utilize NAm as the pyridine nucleotide as well as NAD, NMN, and NR, but the V-factor-dependent strains cannot. This distinction between V-factor-dependent and -independent strains based on growth on complex media is somewhat artificial, since most complex media contain significant amounts of NAm (17, 18). Niven and O'Reilly (18) proposed that the distinction between V-factor-independent and -dependent strains in the family *Pasteurel-*

laceae may reflect the presence or absence of a single enzyme, NAm-PRTase, to convert NAm to NMN. Our results support this proposal. We used the ability to grow on a complex medium without added V-factor as our selection for *nadV* and demonstrated that the presence of *nadV* could also confer the ability to grow on a chemically defined medium to which NAm had been added, but not on the same medium with no exogenous source of pyridine nucleotide. We further demonstrated that NAD could be synthesized from NAm and PRPP via NMN in *A. pleuropneumoniae* strains containing the *nadV* gene, which supports the conclusion that this gene encodes an NAm-PRTase. In addition, in an analysis of currently available genomic databases, we found homologues of NadV in *P. multocida* and *A. actinomycetemcomitans*, two V-factor-independent species, but failed to find a homologue in *H. influenzae*, which is V-factor dependent.

We also identified homologues of NadV in genomes from a variety of highly diverse bacterial species, including two mycoplasmas, a cyanobacterium, a gram-negative aquatic and soil bacterium, and a gram-positive radiation-resistant coccus. The *H. ducreyi nadV* gene was more closely related to the homologues found in *Shewanella* and in *Mycoplasma* species than to either the *P. multocida* or *A. actinomycetemcomitans* homologue. This likely indicates that horizontal transfer of this gene has occurred. The *nadV* gene is located on a plasmid in *H. ducreyi* but in the chromosome of the other bacterial species. One possibility is that this gene moved into *H. ducreyi* from *M. genitalium*. A similar horizontal transfer has been proposed as the source of the *tetM* gene found in most urogenital pathogens of humans (23). We did not find NadV homologues in a wide variety of other species, including members of the *Enterobacteriaceae* and *Bacillaceae*, known to either synthesize NAD de novo or possess pyridine salvage pathways.

The only homologue of NadV with a proposed function to date is human PBEF (24). The human PBEF gene was transcribed mainly in human bone marrow, liver, and muscle cells as well as in activated human lymphocytes. It was proposed to encode a novel cytokine-like molecule that enhanced the effect of stem cell factor and interleukin-7 on B-cell development, but this has not been studied further. The function of NadV in the biosynthesis of NAD should provide an important clue to the role of PBEF in mammalian species.

To date, NadV homologues identified in microbial genome sequencing projects have been designated as homologues of PBEF. For *M. genitalium*, this similarity led to the hypothesis that this gene could be linked to pathogenicity via a potential immune regulatory function (21). Our discovery provides a more plausible explanation for the role of this gene in bacterial metabolism and will be useful in future microbial genome analyses as an indicator of the presence of an alternative NAD biosynthetic pathway.

The requirement for V-factor is a key taxonomic criterion for identification of members of the *Pasteurellaceae*. Our results suggest that the inability to utilize NAm to fill this requirement is due to the absence of a single gene, *nadV*. We have shown that two V-factor-independent species, *P. multocida* and *A. actinomycetemcomitans*, possess chromosomal copies of this gene, while *H. influenzae*, the only V-factor-dependent species for which a complete genome sequence is available, does not possess this gene. The location of the *H. ducreyi nadV* gene on a plasmid and its apparent mobility into other V-factor-dependent species of haemophili suggest that the use of NAD requirements in identification of individual members of the *Pasteurellaceae* could prove problematic in the future. However, at this time NAD independence is not widespread in *H. ducreyi, H. paragallinarum, H. parainfluenzae*, or *A. pleuropneumoniae*, and it seems feasible to continue to use this characteristic as a taxonomic criterion with the caveat that NAD-independent strains of these species do exist.

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REFERENCES

1. **Bragg, R. R., L. Coetzee, and J. A. Verschoor.** 1993. Plasmid-encoded NAD independence in some South African isolates of *Haemophilus paragallinarum*. Onderstepoort J. Vet. Res. **60:**147–152.
2. **Bricker, J., M. H. Mulks, A. G. Plaut, E. R. Moxon, and A. Wright.** 1983. IgA1 proteases of *Haemophilus influenzae*: cloning and characterization in *Escherichia coli* K-12. Proc. Natl. Acad. Sci. USA **80:**2681–2685.
3. **Cynamon, M. H., T. B. Sorg, and A. Patapow.** 1988. Utilization and metabolism of NAD by *Haemophilus parainfluenzae*. J. Gen. Microbiol. **134:**2789–2799.
4. **Foster, J. W., and A. G. Moat.** 1980. Nicotinamide adenine dinucleotide biosynthesis and pyridine nucleotide cycle metabolism in microbial systems. Microbiol. Rev. **44:**83–105.
5. **Fuller, T. E., B. J. Thacker, and M. H. Mulks.** 1996. A riboflavin auxotroph of *Actinobacillus pleuropneumoniae* is attenuated in swine. Infect. Immun. **64:**4659–4664.
6. **Genetics Computer Group.** 1999. Program manual for the Wisconsin package, 10th ed. Genetics Computer Group, Madison, Wis.
7. **Gromkova, R., and H. Koornhof.** 1990. Naturally occurring NAD-independent *Haemophilus parainfluenzae*. J. Gen. Microbiol. **136:**1031–1035.
8. **Hanahan, D.** 1983. Studies on transformation of *Escherichia coli* with plasmids. J. Mol. Biol. **166:**557–580.
9. **Herriott, R. M., E. M. Meyer, and M. Vogt.** 1970. Defined nongrowth media for stage II development of competence in *Haemophilus influenzae*. J. Bacteriol. **101:**517–524.
10. **Holloway de Corsier, M. A.** 1994. Ph.D. thesis. University of Berne, Berne, Switzerland.
11. **Kasarov, L. B., and A. G. Moat.** 1973. Biosynthesis of NAD in *Haemophilus haemoglobinophilus*. Biochim. Biophys. Acta **320:**372–378.
12. **Kilian, M.** 1976. A taxonomic study of the genus *Haemophilus*, with the proposal of a new species. J. Gen. Microbiol. **93:**9–62.
13. **Kilian, M., and E. L. Biberstein.** 1984. Genus II: *Haemophilus*, p. 558–575. *In* N. R. Krieg and J. G. Holt (ed.), Bergey's manual of systematic bacteriology, vol. 1. The Williams and Wilkins Co., Baltimore, Md.
14. **Michelli, V., and S. Sestini.** 1997. Determining NAD synthesis in erythrocytes. Methods Enzymol. **280:**211–221.
15. **Miflin, J. K., R. F. Horner, P. J. Blackall, X. Chen, G. C. Bishop, C. J. Morrow, T. Yamaguchi, and Y. Iritani.** 1995. Phenotypic and molecular characterization of V-factor (NAD)-independent *Haemophilus paragallinarum*. Avian Dis. **39:**304–308.
16. **Morse, S. A., and L Bartenstein.** 1980. Purine metabolism in *Neisseria gonorrhoeae*: the requirement for hypoxanthine. Can. J. Microbiol. **26:**13–20.
17. **Niven, D., and M. Levesque.** 1988. V-factor-dependent growth of *Actinobacillus pleuropneumoniae* biotype 2 (Bertschinger 2008/76). Int. J. Syst. Bacteriol. **38:**319–320.
18. **Niven, D. F., and T. O'Reilly.** 1990. Significance of V-factor dependency in the taxonomy of *Haemophilus* species and related organisms. Int. J. Syst. Bacteriol. **40:**1–4.
19. **O'Reilly, T., and D. F. Niven.** 1986. Defining the metabolic and growth responses of porcine haemophili to exogenous pyridine nucleotides and precursors. J. Gen. Microbiol. **132:**807–818.
20. **O'Reilly, T., and D. F. Niven.** 1986. Pyridine nucleotide metabolism by extracts derived from *Haemophilus parasuis* and *H. pleuropneumoniae*. Can. J. Microbiol. **32:**733–737.
21. **Ouzounis, C., G. Casari, A. Valencia, and C. Sander.** 1996. Novelties from the complete genome of *Mycoplasma genitalium*. Mol. Microbiol. **20:**898–900.

22. **Pohl, S., H. U. Bertschinger, W. Frederiksen, and W. Mannheim.** 1983. Transfer of *Haemophilus pleuropneumoniae* and the *Pasteurella haemolytica*-like organism causing porcine necrotic pleuropneumonia to the genus *Actinobacillus (Actinobacillus pleuropneumoniae* comb. nov.) on the basis of phenotypic and deoxyribonucleic acid relatedness. Int. J. Syst. Bacteriol. **11:**510–514.
23. **Roberts, M. C., S. L. Hillier, J. Hale, K. K. Holmes, and G. E. Kenny.** 1986. Tetracycline resistance and *tetM* in pathogenic urogenital bacteria. Antimicrob. Agents Chemother. **30:**810–812.
24. **Samal, B., Y. Sun, G. Stearns, C. Xie, S. Suggs, and I. McNiece.** 1994. Cloning and characterization of the cDNA encoding a novel human pre-B-cell colony-enhancing factor. Mol. Cell. Biol. **14:**1431–1437.
25. **West, S. E., M. J. Romero, L. B. Regassa, N. A. Zielinski, and R. A. Welch.** 1995. Construction of *Actinobacillus pleuropneumoniae-Escherichia coli* shuttle vectors: expression of antibiotic-resistance genes. Gene **160:**81–86.
26. **Windsor, H. M., R. C. Gromkova, and H. J. Koornhof.** 1993. Transformation of V-factor independence from *Haemophilus ducreyi* to *Haemophilus influenzae* and *Haemophilus parainfluenzae*. Med. Microbiol. Lett. **2:**159–167.
27. **Windsor, H. M., R. C. Gromkova, and H. J. Koornhof.** 1991. Plasmid-mediated NAD independence in *Haemophilus parainfluenzae*. J. Gen. Microbiol. **137:**2415–2421.
28. **Windsor, H. M., R. C. Gromkova, and H. J. Koornhof.** 1993. Growth characteristics of V-factor-independent transformants of *Haemophilus influenzae*. Int. J. Syst. Bacteriol. **434:**799–804.