Microbiology (2015), **161**, 2127–2136 DOI 10.1099/mic.0.000164

Characterization of NAD salvage pathways and their role in virulence in *Streptococcus pneumoniae*

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Received 4 March 2015
Accepted 21 August 2015

NAD is a necessary cofactor present in all living cells. Some bacteria cannot *de novo* synthesize NAD and must use the salvage pathway to import niacin or nicotinamide riboside via substrate importers NiaX and PnuC, respectively. Although homologues of these two importers and their substrates have been identified in other organisms, limited data exist in *Streptococcus pneumoniae*, specifically, on its effect on overall virulence. Here, we sought to characterize the substrate specificity of NiaX and PnuC in *Str. pneumoniae* TIGR4 and the contribution of these proteins to virulence of the pathogen. Although binding affinity of each importer for nicotinamide mononucleotide may overlap, we found NiaX to specifically import nicotinamide and nicotinic acid, and PnuC to be primarily responsible for nicotinamide riboside import. Furthermore, a *pnuC* mutant is completely attenuated during both intranasal and intratracheal infections in mice. Taken together, these findings underscore the importance of substrate salvage in pneumococcal pathogenesis and indicate that PnuC could potentially be a viable small-molecule therapeutic target to alleviate disease progression in the host.

INTRODUCTION

NAD is an essential cofactor used by all living organisms. All bacterial species use pathways to reduce NAD^+ to NADH, for example respiration, which involves either glycolysis and the tricarboxylic acid cycle or fermentation (Jurtshuk, 1996). Bacteria also use NAD^+ in several forms of dehydrogenases when breaking down aldehydes and alcohols (Kotrbova-Kozak *et al.*, 2007; Luong *et al.*, 2015; Nobelmann & Lengeler, 1996; Temple *et al.*, 1994). NAD is utilized in a number of cellular processes in both bacterial and mammalian cells, for example DNA ligation and repair, redox recycling in the pyruvate dehydrogenase pathway, and synthesis of acetyl-CoA for the tricarboxylic acid cycle (Chalkiadaki & Guarente, 2012; Chiarugi *et al.*, 2012; Ishino *et al.*, 1986; Patel *et al.*, 2014; Satoh & Lindahl, 1992; Wilkinson *et al.*, 2001). To regenerate NAD^+, NADH dehydrogenase, part of the NADH: ubiquinone oxidoreductase complex, oxidizes NADH in the bacterial electron transport chain, using the electrons to form a proton gradient that is later used to synthesize ATP (Braun *et al.*, 1998; Friedrich, 1998).

In bacteria, NAD synthesis is a tightly regulated intracellular process (Huang *et al.*, 2009). There are two major ways that bacteria obtain the essential cofactor NAD: through *de novo* synthesis, which occurs in *Escherichia coli* and multiple members of the genera *Bacillus* and *Clostridium*, and through the salvage pathway, which occurs in the order *Lactobacillales*, specifically in members of the family *Streptococcaceae*. Some bacteria, such as *Salmonella enterica* serovar Typhimurium, have both systems (Chandler & Gholson, 1972; Rodionov *et al.*, 2008; Sorci *et al.*, 2013; Spector *et al.*, 1985; Zhu *et al.*, 1988). While the *de novo* pathway is characterized by synthesizing NAD from the amino acid aspartic acid, the salvage pathway imports intermediates several steps downstream in the NAD *de novo* synthesis pathway (Rodionov *et al.*, 2008). The two major importers in the NAD salvage pathway are NiaX, responsible for niacin uptake, and PnuC, responsible for nicotinamide riboside (Herbert *et al.*, 2003; Rodionov *et al.*, 2008, 2009; Rodionov *et al.*, 2008, 2009; Sauer *et al.*, 2004).

Streptococcus pneumoniae is the causative agent of pneumonia, meningitis, otitis media, and bacteraemia leading to sepsis. Although there are several capsule-based vaccines against *Str. pneumoniae*, these vaccines protect against only a subset of over 90 known serotypes. Furthermore, antibiotic resistance within the pneumococcal population can disseminate rapidly owing to the bacterium's ability to quickly exchange resistance determinants through natural competence, a problem that is especially prevalent with beta-lactam antibiotic resistance (Chewapreecha *et al.*, 2014). The rise of antibiotic resistance is leading to the

Abbreviation: SOE-PCR, splicing by overhang extension method of PCR.

necessity for new bacterial targets for small-molecule therapeutics, particularly for novel targets that are essential during infection.

Because of the importance of NAD biosynthesis in bacterial physiology and the potential accessibility to the NAD salvage molecule importers on the bacterial surface, we wanted to assess their functional role in both uptake and virulence in the pneumococcus. Previous studies in *Haemophilus influenzae* found these substrates important for survival in the blood and the PnuC pathway essential during infection (Herbert *et al.*, 2003; Schmidt-Brauns *et al.*, 2001). Here, by making single deletions of the NAD salvage importer genes, we have confirmed which precursors in NAD synthesis are imported by NiaX or PnuC in *Str. pneumoniae* and determined the effect of these mutations on virulence in murine models of pneumococcal infection.

METHODS

Bacterial constructs. Mutations of SP_1233 (*niaX*) and SP_1859 (*pnuC*) were created by using the splicing by overhang extension method of PCR (SOE-PCR). Briefly, 1 kb fragments upstream and downstream of the target gene were amplified and spliced to an erythromycin (Erm) resistance cassette. SOE-PCR products were subsequently transformed into the TIGR4 strain of *Str. pneumoniae*. Knockout mutants selected by antibiotic resistance to Erm were verified by PCR to confirm insertion of the SOE-PCR product and deletion of the target gene (Horton *et al.*, 1990). To complement the mutant strains, the coding regions for *niaX* and *pnuC* were amplified from TIGR4 genomic DNA using primers NiaX_F/NiaX_R (GGCG CGAATTCGGAGGACAAACATGTCTGGTTTATTGTACCATACTA GTGTATATGC, GATCCTGCAG-TTAACGGCGTTTTCGAAGCACT) and PnuC_F/PnuC_R (GCCGGCGGAATTCGGAGGACAAACATGA TGCATACTTATTTGCAAAAGAAAATTG, GGCGCTGCAGCTAGTT AAGTAAATCAGTATTCTGC), respectively; the conserved RBS (GGAGGACAAAC) was included in the forward primer to afford better expression. Amplified *niaX* and *pnuC* were then digested with *Eco*RI/*Pst*I and ligated with streptococcal shuttle vector pABG5 (Granok *et al.*, 2000). The generated plasmids pABG5-*niaX* and pABG5-*pnuC* were transformed into TIGR4Δ*niax* and TIGR4Δ*pnuc*, respectively, using standard transformation protocols. Sequence insertion in the complemented strains was confirmed using plasmid-specific primers.

Growth curves. Bacteria were grown at 37 °C with 5 % CO_2 in C+Y medium to an OD_{620} of 0.1, centrifuged, and resuspended in C+Y without nicotinic acid, nicotinamide, or yeast (C−Y). Bacterial strains were incubated at 37 °C with 5 % CO_2 for 2 h, then back-diluted to an OD_{620} of 0.1 in C−Y with 20 % v/v glycerol and frozen for initial stock solutions. Stocks were titrated to assure equal starting bacterial numbers. Assays were performed in a Cytation 3 cell imaging multimode reader (BioTek) using a 96-well format. Stock solutions were back-diluted 1 : 40 for growth curves in C+Y, C−Y, or C−Y medium supplemented with the NAD salvage substrates nicotinic acid (Sigma), nicotinamide mononucleotide (Sigma), nicotinamide (Sigma), or nicotinamide riboside (ChromaDex); all growth curves were performed in quadruplicate. The OD_{620} of samples was read every 30 min, with a 5 s double orbital shake before each read.

Radiolabelled uptake. For uptake experiments, strains were cultured in standard C+Y to OD_{620} 0.4 and then subjected to a 1 : 10 back dilution and outgrown in C−Y during labelling. Substrates utilized were nicotinic acid (^{14}C-carbonyl; 55 mCi (1035 MBq) mmol^{-1}, nicotinamide (^{14}C-carbonyl; 55 mCi mmol^{-1}) and nicotinamide β-riboside triflate salt (^{14}C-carbonyl; 55 mCi mmol^{-1}). All ^{14}C-labelled substrates were purchased from American Radiolabelled Chemicals (St Louis, MO). ^{14}C-Labelled substrates were added at a final concentration of 1 μM for nicotinamide β-riboside and nicotinic acid, and to a final concentration of 500 nM for nicotinamide, all in 1 ml volume of culture. Cultures were subsequently incubated at 37 °C for 30 min to allow uptake of the respective substrates. Cells were collected onto a 0.45 μM pore filter disc (Millipore) by vacuum filtration and the membrane was washed with 5 ml PBS, also via vacuum filtration. Dried filters were resuspended in 3 ml ScintiSafe (Fisher Scientific) and the counts min^{-1} of the respective samples were detected for 1 min in a Perkin Elmer liquid scintillation counter. For competition experiments, experimental conditions were identical, with the exception of the addition of 5 μM nicotinamide mononucleotide with 1 μM respective substrates. For all experiments, uptake was normalized to the counts min^{-1} of the parental WT TIGR4 strain, which was considered to have an uptake of 100 % for all substrates.

Mouse studies. All experiments involving animals were performed with prior approval of and in accordance with guidelines of the St Jude Institutional Animal Care and Use Committee. The St Jude laboratory animal facilities have been fully accredited by the American Association for Accreditation of Laboratory Animal Care. Laboratory animals were maintained in accordance with the applicable portions of the Animal Welfare Act and the guidelines prescribed in the DHHS publication, *Guide for the Care and Use of Laboratory Animals*. All mice were maintained in BSL2 facilities and all experiments were done while the mice were under inhaled isoflurane (2.5 %) anaesthesia. Mice were monitored daily for signs of infection. This work was approved under the IACUC protocol number 538-100013-04/12 R1.

For survival studies, cultured bacteria were grown in C+Y medium to an OD_{620} of 0.4 and diluted according to a previously determined standard curve. Bacteria were plated to assure that the proper amount of bacteria was added. For intranasal infections, bacteria were introduced into 6-week-old BALB/c mice (Jackson Laboratory) via intranasal administration of 10^7 c.f.u. bacteria in PBS (25 μl). Blood for titre determination was collected via tail snip at both 24 and 48 h post-infection. Survival data were analysed by using the Mantel–Cox log rank test in Prism 6. For intratracheal infections, mice were infected via intranasal administration with 10^5 c.f.u. bacteria in PBS (100 μl). For lung collection, lungs were washed twice with PBS to remove contaminating blood. The lungs were then homogenized and plated for c.f.u. titres. Blood from the chest cavity was also collected for c.f.u. titres.

Homology. All protein sequences were aligned using NCBI BLAST (Altschul *et al.*, 1990, 1997). Sequences were analysed for overall protein identity.

RESULTS

Homology of NiaX and PnuC

Str. pneumoniae contains homologues of both NiaX and PnuC, but there is limited knowledge regarding both importers specific to the organism. Interestingly, some streptococcal species do not have any proteins that are homologous to NiaX but do share homology to pneumococcal PnuC (Fig. 1a). In both cases, there is limited homology outside the respective *Streptococcus* genus, suggesting the proteins studied here could potentially yield different

(a) *Str. pneumoniae* NAD salvage importer homology compared with TIGR4 isolate

Strain	PnuC protein identity (%)	Nilax protein identity (%)	Gram stain
Streptococcus mitis	98	96	+
Streptococcus oralis	97	90	+
Streptococcus sanguinis	89	60	+
Streptococcus sabrinus	75	No homology	+
Streptococcus gordonii	57	63	+
Streptococcus downei	74	No homology	+
Streptococcus pyogenes	30	41	+
Haemophilus influenzae	37	No homology	−
Clostridium clariflavum	31	No homology	+
Neisseria mucosa	**32**	No homology	−
Enterococcus faecalis	31	42	+

(b)

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TIGR4        79   DWIGMICSLTGIICVIFVSEGRASNYLFGLINSVIYLILALQKGFYGE-VLTTLYFTVMQ  137
                   W+  + ++TGI+CV+FV +G+ SNYLFGLI+  +Y  ++     YGE  L  L +  +Q
N. mucosa    40   SWLASVAAVTGILCVVFVGKGKISNYLFGLISVSLYAYVSYTFKLYGEMMLNLLVYVPVQ  99

TIGR4       138   PIGLLVWIYQAQFKK--EKQEFVARKLDGKGWTKYLSISVLWWLAFGFIYQSIGANRPYR  195
                   +G   W       +  E +E  A+ L  + W   ++ SV+    +      +G+  P
N. mucosa   100   FVGFAMWRKHMALGETAETEEVKAKALTVRQWLLVVAASVVGTSVYIEWLHHLGSALPTL  159

TIGR4       196   DSITDATNGVGQILMTAVYREQWIFWAATNVFSIYLW---W---GE-SLQIQGKYLIYLI  248
                   D +T   + V Q+LM   YREQW  W   N+ +I LW   W   GE SL +   Y++YL
N. mucosa   160   DGVTVVVSIVAQVLMILRYREQWALWIVVNILTISLWAVAWFKNGETSLPLLLMYVMYLC  219

TIGR4       249   NSLVGWYQWSKAAKQNT  265
                  NS+ G+  W+K  K+++
N. mucosa   220   NSVYGYINWTKLVKRHS  236
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Fig. 1. PnuC and NiaX homology with known sequences and structures. (a) The TIGR4 PnuC and NiaX protein sequences were compared with other bacterial PnuC and NiaX homologues by using a BLAST search (Altschul *et al.*, 1997); percentage identities are listed together with Gram stain results. (b) Structural alignment of TIGR4 PnuC and *Neisseria mucosa* PnuC (Jaehme *et al.*, 2014), for which the structure was solved with nicotinamide riboside coordinated within the protein. Residues involved in nicotinamide riboside binding, the cytoplasmic gate, both the cytoplasmic gate and nicotinamide riboside binding, and the periplasmic gate are highlighed in red, blue, purple, and orange, respectively.

results from other orthologues. The solved structure of PnuC bound to nicotinamide riboside in *Neisseria mucosa* identified the nicotinamide riboside-binding residues, cytoplasmic gate and periplasmic gate. All but 2 of the 17 residues were identical in *Str. pneumoniae*; those two (T130 in TIGR4 versus N91 in *N. mucosa* and N203 in TIGR4 versus S168 in *N. mucosa*) contained functionally similar side chains (−OH) and were not directly responsible for coordinating nicotinamide riboside (Fig. 1b) (Jaehme *et al.*, 2014).

Preparation and testing of NAD depletion medium

C+Y is a synthetic medium for growth of *Str. pneumoniae* (Lacks & Hotchkiss, 1960). For growth-curve testing of the NAD salvage pathway substrates, all known sources of imported precursors had to be excluded from the medium, including nicotinic acid (niacin), nicotinamide and yeast, which is a common source of nicotinic acid, nicotinamide and other molecules that the bacteria could use in the NAD salvage pathway. This medium, termed C−Y, was assessed as a growth medium for *Str. pneumoniae*. There was no growth of the WT TIGR4 or of the Δ*niaX* and Δ*pnuC* mutants, indicating the successful depletion of necessary NAD pathway precursors (Fig. 2). Additionally, all strains underwent equal exponential growth in the C+Y medium, signifying that the mutant strains had no initial growth defect; however, for unknown reasons, the Δ*pnuC* mutant seemed to undergo autolysis more rapidly than either the WT or Δ*niaX* mutant strains did. This effect was not attributable to pH as the culture's pH was indistinguishable from that of WT following overnight growth (data not shown).

Substrate characterization of NAD salvage pathway importers

To assess import of the substrates of the NAD salvage pathway, C−Y was supplemented with nicotinic acid, nicotinamide, and nicotinamide riboside. In each experiment, TIGR4 and the mutants were grown in C−Y or C+Y as negative and positive controls, respectively (data not

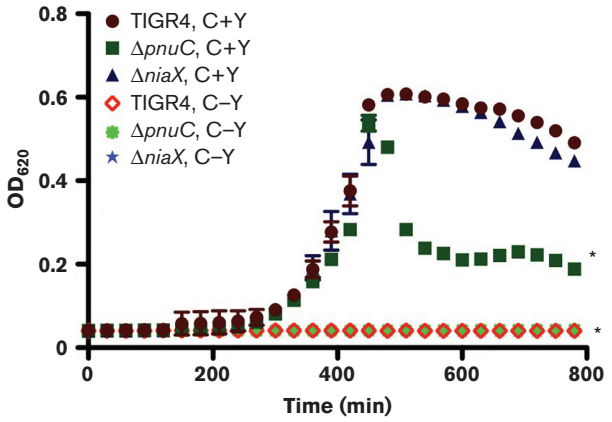

Fig. 2. C−Y is a viable medium for studying components of the NAD salvage pathway. TIGR4, Δ*niaX*, and Δ*pnuC* grown in C+Y were compared with the same strains grown in C−Y by using a one-way ANOVA and by Dunn's multiple comparison post-test using TIGR4 in C+Y as the basis of comparison (n=4). *, $P < 01$.

shown). In nicotinic acid- and nicotinamide-supplemented media, the Δ*pnuC* mutant grew as well as TIGR4 (Fig. 3b, d) while the Δ*niaX* mutant showed a growth defect (Fig. 3a, c), which was restored upon complementation. This result confirmed previous predictions and *in vitro* data about the target of NiaX and, we believe, provided the first *in vivo* data supporting NiaX being the nicotinic acid/nicotinamide importer in bacteria, specifically in *Str. pneumoniae* (Rodionov *et al.*, 2009; ter Beek *et al.*, 2011).

Nicotinamide riboside was the next compound to be tested as an NAD salvage pathway substrate. The Δ*pnuC* mutant had poor growth in the C−Y medium supplemented with nicotinamide riboside compared with the Δ*niaX* mutant, which showed WT levels of growth (Fig. 3e, f), a phenotype that was restored upon complementation. This result is in agreement with previous reports that PnuC imports nicotinamide riboside into the related bacterium *Streptococcus pyogenes,* indicating PnuC may have similar substrate specificity amongst other streptococci (Sauer *et al.*, 2004).

To confirm substrate transport capacity of these transporters, we measured the relative uptake of the radiolabelled substrates. Corroborating the growth-curve experiments, uptake of both nicotinamide and nicotinic acid were reduced in the Δ*niaX* mutant, a phenotype restored to WT levels upon complementation of *niaX* (Fig. 4a, b). The Δ*pnuC* mutant retained the capacity to import both nicotinamide and nicotinic acid (data not shown), but was severely impaired in the acquisition of nicotinamide riboside, a defect ameliorated when *pnuC* was complemented on a plasmid (Fig. 4c). It should be noted that greater concentrations of labelled substrates than that used in the growth-curve experiments were utilized in order to achieve sufficient label incorporation. Taken together, these data confirm the substrate specificity of these importers in the pneumococcus.

In addition to these typical substrates, we also assessed the capacity of nicotinamide mononucleotide to rescue pneumococcal growth. Import of this substrate has previously been shown to be dependent on a phosphorylase such as AphA (*Salmonella*) or NadN (*Hae. influenzae*) (Grose *et al.*, 2005; Kemmer *et al.*, 2001). Both the Δ*niaX* and the Δ*pnuC* mutants underwent WT growth in C−Y supplemented with nicotinamide mononucleotide (Fig. 5a), suggesting that either both proteins can import the molecule, which is modified at the cell surface and subsequently imported by either of these systems, or an additional import machinery exists. To address this unexpected finding, we assessed the capacity of exogenously added nicotinamide mononucleotide to compete with nicotinamide, nicotinic acid, and nicotinamide riboside for uptake. Interestingly, supplementation of the culture with a molar ratio of five nicotinamide mononucleotide to one of any of the other three substrates partially blocked uptake of all three of the radiolabelled substrates (Fig. 5b).

Virulence of NAD salvage pathway importers in mice

Because NAD is an essential cofactor, we hypothesized that the salvage pathways would be required for pneumococci to acquire NAD precursors from the mammalian host and, consequently, for their survival and pathogenicity, as seen previously with the PnuC salvage system in *Hae. influenzae in vivo* studies (Herbert *et al.*, 2003). To test this, mice were intranasally infected with *Str. pneumoniae* (which progresses rapidly from the sinuses to the lungs and eventually traverses into the bloodstream) WT TIGR4, the Δ*niaX* mutant or the Δ*pnuC* mutant, and then mouse survival and blood titres were assessed. The virulence of the salvage pathway import mutants varied from each other, with the Δ*niaX* mutant having WT levels of pathogenesis and the Δ*pnuC* mutant being completely attenuated in the intranasal model (Fig. 6a–c). Further investigation of the Δ*pnuC* mutant in an intratracheal pneumonia model yielded the same attenuation phenotype as WT TIGR4, as measured by blood and lung titres (Fig. 6d, e). Taken together, these data indicate that PnuC is critical for lung colonization and bacterial virulence in mice whereas NiaX is dispensable.

DISCUSSION

Here, we sought to use both *in vitro* and *in vivo* techniques to confirm substrates of the NiaX and PnuC importers and assess the role of these systems during invasive pneumococcal disease. Our data confirmed that NiaX showed preference for importing nicotinic acid and nicotinamide, that PnuC specifically imported nicotinamide riboside, and that single deletions of both genes retained capacity to

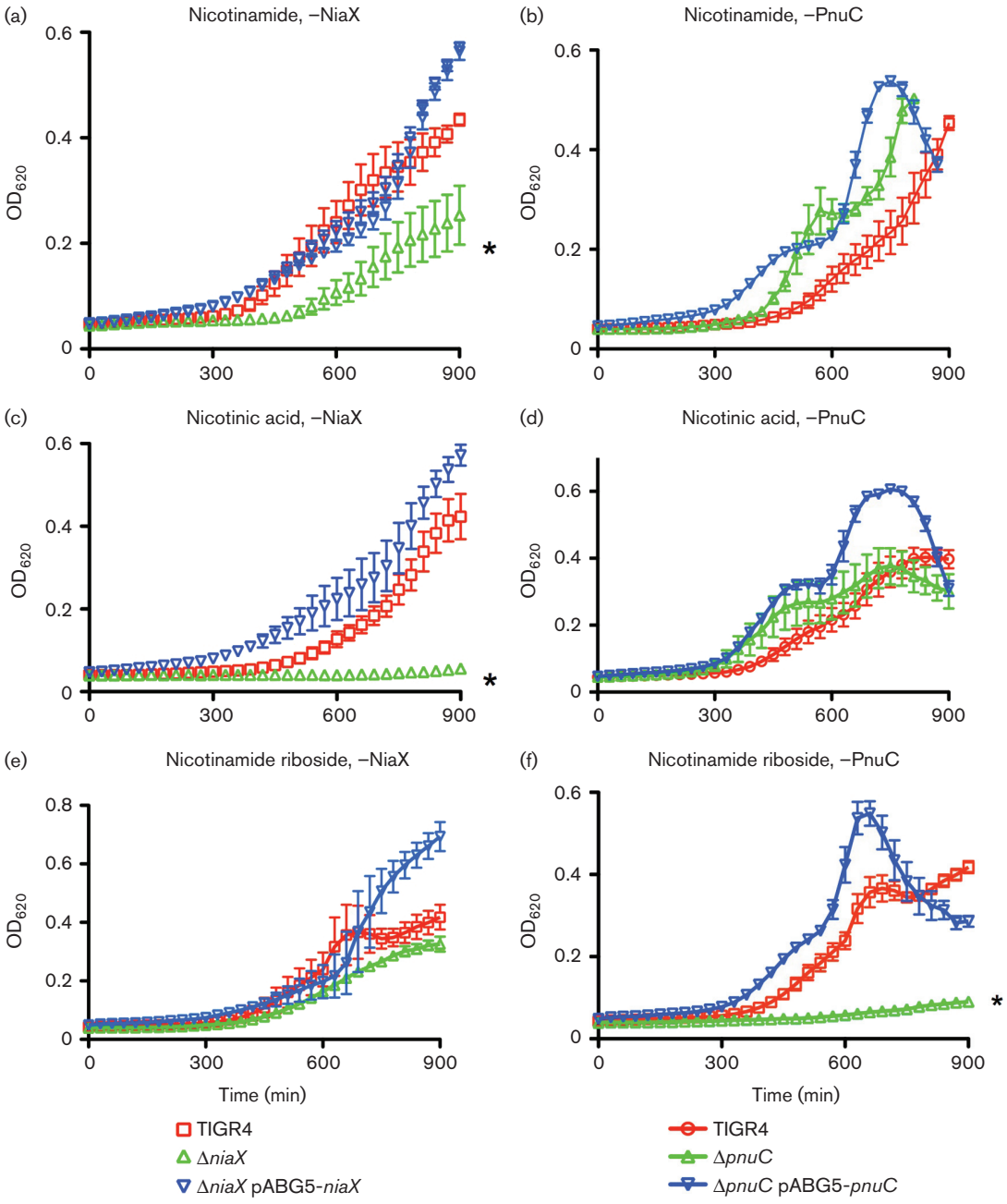

Fig. 3. NAD salvage substrate importer mutants are recovered by specific salvage pathway compounds. Mutant strains Δ*niaX* and Δ*pnuC* were grown in C−Y supplemented with 100 nM nicotinamide (a, b), 100 nM nicotinic acid (c, d) and 500 nM nicotinamide riboside (e, f). One-way ANOVA and Dunn's multiple comparison post-test were performed using TIGR4 in the related condition as the basis of comparison (n=4). *, P<01.

import nicotinamide mononucleotide. In the process, we also have shown that the salvage pathway is essential for pneumococcal growth as the C–Y medium contains tryptophan and other components needed for traditional *de novo* synthesis. As the bacteria listed in Fig. 1a are pathogenic, there seems to be no connection between pathogenicity and having the salvage pathway involving NiaX or PnuC alone or involving both importers. Additionally, there seems to be no larger connection between pathogenicity in bacteria that contain *de novo* synthesis alone (*Helicobacter pylori*), both the *de novo* and salvage pathways (*Bacillus anthracis*), or an alternative *de novo*/salvage pathway (*Francisella tularensis*) (Huang *et al.*, 2008; Sorci *et al.*, 2009). The multitude of strategies utilized by these

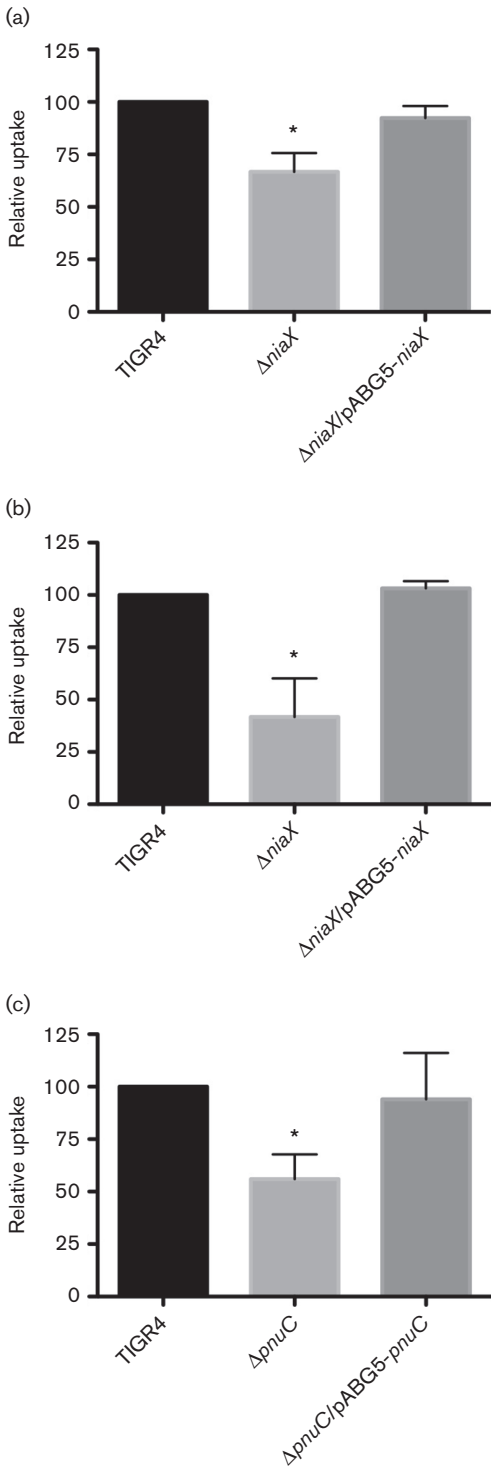

Fig. 4. Uptake of NAD salvage pathway compounds. (a) Nicotinamide, (b) nicotinic acid, (c) nicotinamide riboside. The parental TIGR4 and the Δ*niaX* and Δ*pnuC* mutants along with the complemented strains were grown in C−Y supplemented with ^{14}C-labelled versions of their respective substrates. For Δ*niaX* this was 1 μM nicotinic acid or 500 nM nicotinamide, and for Δ*pnuC* this was 1 μM nicotinamide riboside. After incubation, cells were collected and washed, and the level of uptake was measured. Data were normalized to counts min^{-1} of the parental TIGR4 strain, representing 100 % uptake. Data represent the mean and SD from at least three experiments. *, $P<0.05$ by paired student's *t*-test.

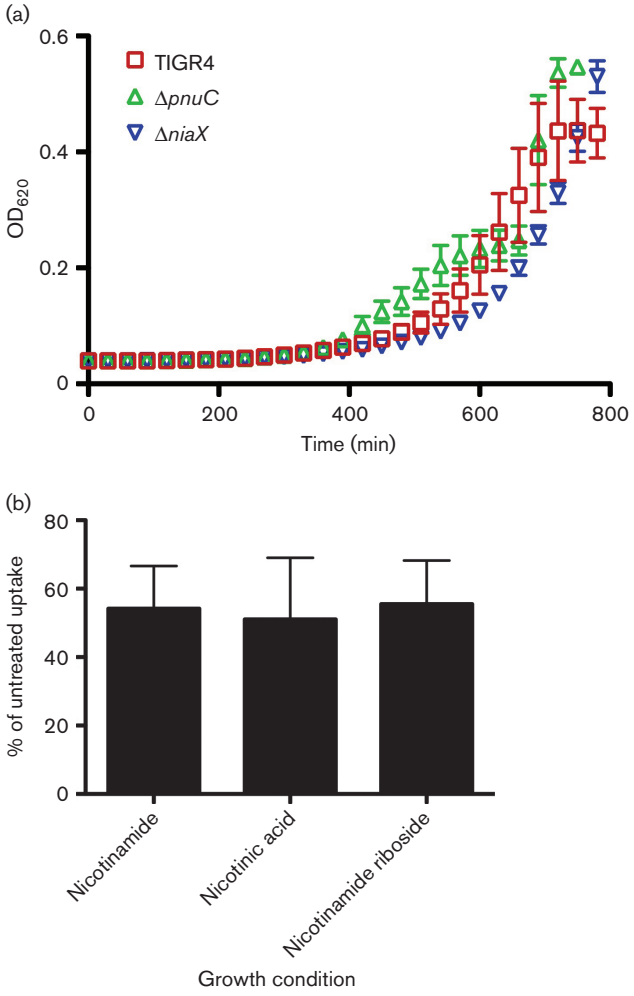

Fig. 5. Nicotinamide mononucleotide rescues growth and inhibits uptake of other NAD salvage pathway components. (a) Parental TIGR4 and Δ*niaX* and Δ*pnuC* mutants were grown in C−Y with and without the addition of 10 μM nicotinamide mononucleotide. One-way ANOVA and Dunn's multiple comparison post-test were performed using TIGR4 in the related condition as the basis of comparison ($n=4$) with no significant differences observed. (b) Competition for uptake was measured by supplementation of nicotinamide mononucleotide to cells grown in C−Y medium with nicotinamide, nicotinic acid, or nicotinamide riboside. Cells were incubated with a 5 : 1 molar ratio of nicotinamide mononucleotide along with the respective ^{14}C-labelled substrates, and measured for uptake after 1 h. Data were normalized to counts min^{-1} of the parental TIGR4 strain, representing 100 % uptake. Data represent the mean and SE from three replicates.

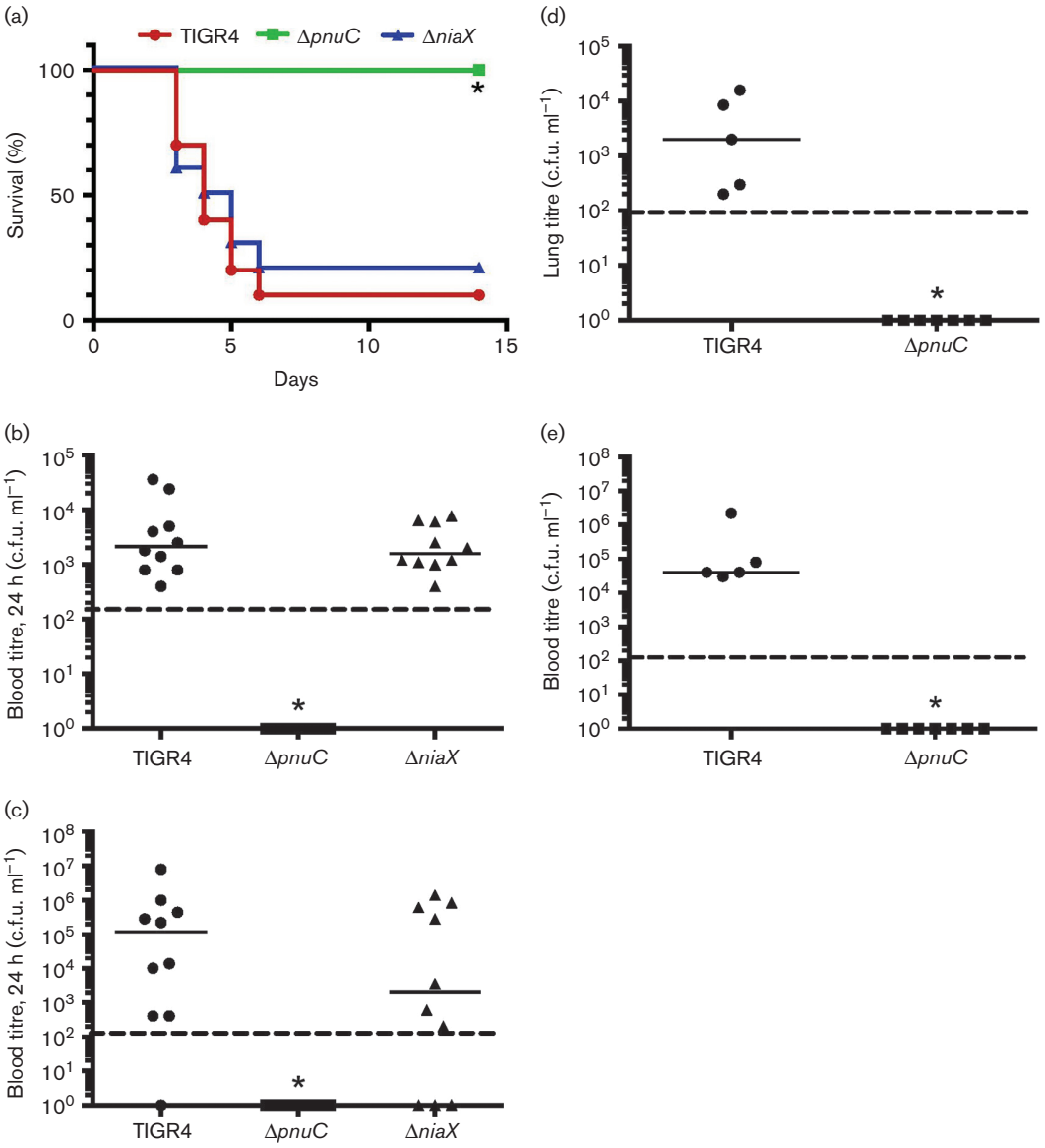

Fig. 6. PnuC is necessary for virulence in a mouse model. (a–c) Mice were intranasally infected with TIGR4 or the Δ*pnuC* or Δ*niaX* mutant and survival (a) and blood titres at 24 (b) and 48 h (c) were assessed. Dashed line represents limit of detection. Survival of mice infected with mutants was compared with that of those infected with WT TIGR4 by using the Mantel–Cox test; blood titre data were compared by using the Mann–Whitney test. (d, e) Mice were intratracheally infected with TIGR4 or the Δ*pnuC* or Δ*niaX* mutant, and lung (d) and blood (e) titres were assessed at 48 h. Lung and blood titre data were compared using the Mann–Whitney test and an ANOVA non-parametric test with a multiple comparison correction with the Kruskal–Wallace test. *, $P<01$.

pathogens to acquire these essential factors underscores their importance in bacterial physiology.

The nicotinamide mononucleotide data suggest either that there is an additional import system in *Str. pneumoniae* or that both NiaX and PnuC are involved in import, perhaps via an extracellular protein that can modify nicotinamide mononucleotide to an importable form. There is considerable variability amongst PnuC homologues involving nicotinamide riboside binding and homology of critical residues (Jaehme *et al.*, 2014). The PnuC homologues from both *Hae. influenzae* and *Sal. typhimurium* do not import nicotinamide mononucleotide but can convert it to the form importable by PnuC (nicotinamide riboside) via NadN or AphA, respectively. The PnuC from these two organisms and from *Str. pneumoniae* all contain the motif for nicotinamide mononucleotide binding. However, PnuC homologues from several other organisms do not contain the consensus binding residues (Grose *et al.*,

2005; Kemmer *et al.*, 2001; Sauer *et al.*, 2004). This observation suggests that separate classes of NAD salvage substrate importers annotated as PnuC import nicotinamide riboside and/or nicotinamide mononucleotide and that NiaX (where not enough structural information is known) imports niacin and/or nicotinamide mononucleotide as preferred substrates. This hypothesis is supported by the sequence divergence in both TIGR4 NiaX and PnuC as compared with sequences outside the genus. Such amino acid differences would likely impact the overall fold of the respective protein structures and perhaps add the ability to import nicotinamide mononucleotide directly, as observed with mutation of *Salmonella* PnuC (Grose *et al.*, 2005). Interestingly, the amino acids in *Salmonella* PnuC preventing this transporter from importing nicotinamide mononucleotide are not conserved in the pneumococci, indicating that the pneumococcal PnuC may be permissive for this substrate along with nicotinamide riboside. This underscores the importance of experimental confirmation of highly divergent orthologues for substrate specificity in different species.

Although it is possible for both PnuC and NiaX in *Str. pneumoniae* to acquire the ability to import nicotinamide mononucleotide, another possibility is the existence of an additional importer that has not been characterized. Unfortunately, to date, there are no known specific importers of nicotinamide mononucleotide in bacteria. Furthermore, *Str. pneumoniae* does not have an NadN or AphA homologue, which would be used to dephosphorylate nicotinamide mononucleotide to nicotinamide riboside, a form readily imported by PnuC.

On the basis of previous works, sequence homology, and our data described here, we propose the following pneumococcal pathway of NAD synthesis (Fig. 7). NiaX can import either nicotinic acid or nicotinamide, which can be deamidated to nicotinic acid by nicotinamidase PncA (SP_1583). Nicotinic acid phosphoribosyltransferase, PncB (SP_1421), adds a phosphylated ribose to nicotinic acid to form nicotinic acid (nicotinate) mononucleotide. NadD, a nicotinamide/nicotinic acid nucleotide adenylyltransferase (SP_1747), then adds an adenine, forming nicotinic acid adenine dinucleotide. The NAD synthase NadE (SP_1420) then adds the final amide group to form NAD. NAD synthesis via nicotinamide riboside occurs in considerably fewer steps than does NAD synthesis through nicotinic acid or nicotinamide. Once imported, nicotinamide riboside is, in theory, converted to nicotinamide mononucleotide via phosphorylation by an unknown kinase, as the TIGR4 strain of *Str. pneumoniae* does not contain the kinase NadR (Kurnasov *et al.*, 2002; Singh *et al.*, 2002). Then, it is converted to NAD through NadD, which has nucleotide adenylyltransferase activity.

In addition to understanding the substrates of the salvage pathway importers, we sought to analyse their impact on virulence in two mouse infection models. Our data show the attenuation of a pneumococcal Δ*pnuC* mutant in a lung infection model as compared with WT. Interestingly, this result leads to speculation about the bioavailability of these metabolites in the host and to the hypothesis that nicotinamide riboside is more bioavailable than either nicotinic acid or nicotinamide, or that *Str. pneumoniae* is far more efficient in using nicotinamide riboside as a precursor for the NAD salvage pathway than nicotinic acid and nicotinamide, or both.

Nicotinamide riboside supplementation has been implicated in many beneficial functions in the host, including

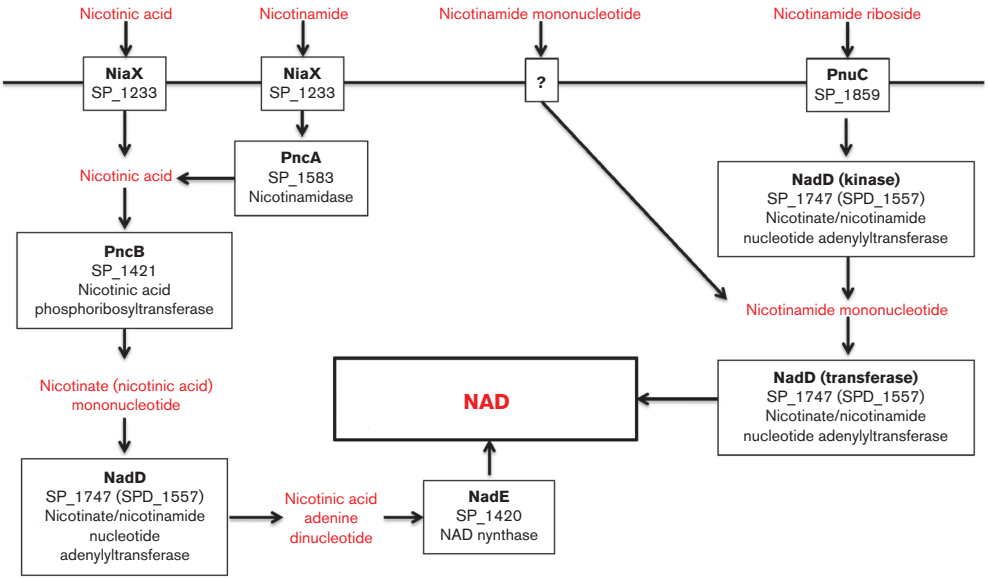

Fig. 7. Proposed NAD pathway in *Str. pneumoniae*.

protection against mitochondrial myopathy (Khan *et al.*, 2014), hearing loss (Brown *et al.*, 2014) and obesity (Cantó *et al.*, 2012), although these functions are not likely due to increasing NAD synthesis (Frederick *et al.*, 2015) but may be due to general bioavailability. Although nicotinamide riboside is necessary for pathogen and host, fortunately pneumococcal PnuC and the homologous proteins of other bacteria have no sequence homology to any proteins in the animal kingdom. Thus, as has been successfully shown with *Hae. influenzae* (Sauer *et al.*, 2004), PnuC could potentially be targeted therapeutically in bacterial species harbouring this pathway without mammalian consequence.

ACKNOWLEDGEMENTS

This work was supported in part by NIAID grant R01AI110618 and by the American Lebanese Syrian Associated Charities (ALSAC). Nicotinamide riboside was a kind gift from ChromaDex.

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Edited by: M. Whiteley