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Carbonic Anhydrase Is Essential for \textit{Streptococcus pneumoniae} Growth in Environmental Ambient Air\textsuperscript{†}

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The respiratory tract pathogen \textit{Streptococcus pneumoniae} needs to adapt to the different levels of carbon dioxide (CO\textsubscript{2}) it encounters during transmission, colonization, and infection. Since CO\textsubscript{2} is important for various cellular processes, factors that allow optimal CO\textsubscript{2} sequestration are likely to be important for pneumococcal growth and survival. In this study, we showed that the putative pneumococcal carbonic anhydrase (PCA) is essential for \textit{in vitro} growth of \textit{S. pneumoniae} under the CO\textsubscript{2}-poor conditions found in environmental ambient air. Enzymatic analysis showed that PCA catalyzes the reversible hydration of CO\textsubscript{2} to bicarbonate (HCO\textsubscript{3}{-}), an essential step to prevent the cellular release of CO\textsubscript{2}. The addition of unsaturated fatty acids (UFAs) reversed the CO\textsubscript{2}-dependent \textit{in vitro} growth inhibition of \textit{S. pneumoniae} strains lacking the pca gene (Δpca), indicating that PCA-mediated CO\textsubscript{2} fixation is at least associated with HCO\textsubscript{3}{-}-dependent de novo biosynthesis of UFAs. Besides being necessary for growth in environmental ambient conditions, PCA-mediated CO\textsubscript{2} fixation pathways appear to be required for intracellular survival in host cells. This effect was especially pronounced during invasion of human brain microvascular endothelial cells (HBMEC) and uptake by murine J774 macrophage cells but not during interaction of \textit{S. pneumoniae} with Detroit 562 pharyngeal epithelial cells. Finally, the highly conserved pca gene was found to be invariably present in both CO\textsubscript{2}-independent and naturally circulating CO\textsubscript{2}-dependent strains, suggesting a conserved essential role for PCA and PCA-mediated CO\textsubscript{2} fixation pathways for pneumococcal growth and survival.

The Gram-positive bacterium \textit{Streptococcus pneumoniae}, or pneumococcus, is a human respiratory tract pathogen that contributes significantly to global mortality and morbidity. In addition, it is an important asymptomatic colonizer of the human nasopharynx, with carriage rates around 10% in adults and over 40% in children (6). Pneumococcal colonization and infection are closely linked, but knowledge of the factors that contribute to transmission, carriage, disease, and transition from carriage to disease is still limited. Research on components that physically contribute to host-pathogen interactions, such as capsule polysaccharides, adhesins, and toxins, has provided valuable insights into the process of pneumococcal pathogenesis (20). In contrast, the influence of environmental factors on pneumococcal growth and survival remains fairly unexplored.

\textit{S. pneumoniae} needs to adapt to various aerobic and anaerobic conditions, reflecting the different niches it occupies during transmission, colonization, and invasive disease. During niche transition, oxygen ($O_2$) levels change considerably. Levels of $O_2$ are 21% in ambient air, decrease to 10 to 15% in the alveoli of the lungs, and are about 5% in resting cells. In O$_2$-rich conditions, \textit{S. pneumoniae} expresses pyruvate oxidase (SpxB), which generates acetyl-phosphate as a source of ATP and hydrogen peroxide ($H_2O_2$) for interspecies competition at the mucosal surfaces of the nasopharynx (41). The presence of $O_2$ is also a prerequisite for the pneumococcal X state (4, 14), which is a physiological condition that allows for genetic transformation and an adequate response to environmental stress (32). Recently, it was shown that the fatty acid (FA) content of the pneumococcal cell membrane (31) and the expression of 69 genes (8) change in response to the availability of $O_2$. Finally, changes in $O_2$ levels can also affect production of the polysaccharide capsule (48), which is the major pneumococcal virulence determinant.

Similar to those of $O_2$, the levels of carbon dioxide (CO$_2$) vary considerably among the different pneumococcal niches inside and outside the host. Ambient levels of CO$_2$ in the environment are 0.038%, while CO$_2$ levels inside the human body, in particular in the lower respiratory tract, can reach 5% or more. The importance of this gaseous compound for \textit{S. pneumoniae} is illustrated by the observation that the depletion of CO$_2$ from ambient air completely inhibits pneumococcal growth (21). Moreover, about 8% of all clinical isolates require a CO$_2$-enriched environment for growth in laboratory conditions (3). This intrinsic CO$_2$ dependence of \textit{S. pneumoniae} and many other (micro)organisms is most likely related to an anabolic need for CO$_2$ or bicarbonate (HCO$_3{-}$) during biosynthesis of nucleic acids, amino acids, and FAs (1). Pathogens can often sequester CO$_2$ directly from host tissues, but in the absence of sufficient levels of extracellular CO$_2$, endogenous CO$_2$ needs to be enzymatically fixed. Carbonic anhydrases...
(CAs; EC 4.2.1.1) are enzymes that catalyze the reversible reaction CO$_2$ + H$_2$O $\leftrightarrow$ HCO$_3^-$ + H$^+$. Because HCO$_3^-$ cannot passively diffuse across biological membranes, its formation significantly delays the release of intracellular CO$_2$. At least five different classes of CAs have been described, and most eukaryotic, prokaryotic, and archaeal species express at least one CA class (39, 40).

Genome analysis (39) has revealed that *S. pneumoniae* has one putative CA, a β-class CA that is highly conserved in all available pneumococcal genome sequences. Pneumococcal CA (PCA) is highly homologous to CAs in other streptococcal species, such as *Streptococcus pyogenes*. The closest nonstreptococcal PCA homologs are found in *Mycobacterium* species, while PCA homologs in other respiratory tract pathogens such as *Neisseria meningitidis* and *Haemophilus influenzae* are more divergent (40). The aim of this study was to investigate the functional characteristics of the *pca* gene and the encoded PCA enzyme in *S. pneumoniae* and to establish the relevance of PCA for pneumococcal growth and survival under CO$_2$-poor conditions *in vitro*. Further, we examined the importance of PCA during host-pathogen interaction.

### MATERIALS AND METHODS

**Bacterial strains and growth conditions.** The bacterial strains that were used in this study are listed in Table 1. *S. pneumoniae* strains were routinely grown under static conditions in GM17 broth (23) or on blood agar (BA) plates composed of Colombia agar (Oxoid) supplemented with 5% sheep blood (Bio- 

trading). Cultures were incubated in a 5%-CO$_2$ incubator at 37°C. To compare growth under CO$_2$-poor and -rich conditions, mid-log-phase cultures of pneumococcal strains in CO$_2$-enriched GM17 were diluted 50-fold in medium that was exposed overnight to ambient air (0.038% CO$_2$) or to ambient air enriched with 5% CO$_2$, respectively. Pneumococcal genetic transformation was performed as described previously (10), and importantly, for preparation of competent *S. pneumoniae* strains lacking the *pca* gene (*Δpca*), all media were first exposed to ambient air enriched with 5% CO$_2$. For transformation of the CO$_2$-dependent carriage strains, a 1:1 mixture of competence-stimulating peptide 1 (CSP-1) (100 ng/ml) and CSP-2 (100 ng/ml) was used. Viable-bacteria counts were derived from CFUs after plating 10-fold serial dilutions in PBS. *Escherichia coli* strains were routinely grown on 37°C on Luria Bertani (LB) agar plates or in LB broth in a shaking incubator at 200 rpm. *E. coli* transformation was performed by the CaCl$_2$ competence method (35). *Lactococcus lactis* strains were routinely grown on GM17 agar plates or in GM17 broth as static cultures at 30°C. *L. lactis* transformation was performed by electroporation (23). The antibiotics and stock solutions used for complementation studies were ampicillin, 100 µg/ml; spectinomycin, 150 µg/ml; kanamycin, 500 µg/ml for *S. pneumoniae* and 50 µg/ml for *E. coli*; trimethoprim, 0.25 µg/ml; chloramphenicol, 2.5 µg/ml for *S. pneumoniae* and 5 µg/ml for *L. lactis*; adenine, 5 µg/ml in 0.05 M HCl; uracil, 2 µg/ml in 1% sodium carbonate (Na$_2$CO$_3$); arginine, 20 µg/ml; asparatic acid, 20 µg/ml (pH 7); palmitic acid or oleic acid, 200 nM in ethanol; sodium salicylate, 1 M; and bovine liver catalase, 200,000 U/ml (Sigma).

**DNA extraction and PCR conditions.** Chromosomal DNA was isolated from *S. pneumoniae* and *E. coli* broth cultures by cetyltrimethylammonium bromide (CTAB) extraction as described previously (47). Plasmids were isolated from *E. coli* and *L. lactis* broth cultures with a Qiagen mini- or midikit (Qiagen). For construction of directed-deletion mutants and glutathione S-transferase (GST) fusion protein cloning, the proofreading *Pwo* DNA polymerase (Roche) was used. For other PCR-based approaches, AmpliTag DNA polymerase (Applied Biosystems) was applied. The primers (Biologo, Nijmegen, Netherlands) that were used in this study are listed in Table S1 in the supplemental material.

**Construction of pneumococcal mutants.** Directed-deletion mutants of *S. pneumoniae* were generated by allelic exchange of the target gene with an antibiotic resistance marker as described previously (10). Briefly, overlap extension PCR was applied to insert the kanamycin or spectinomycin resistance cassette of the R6 mutant derivatives. The overlap extension PCR products were transformed into *S. pneumoniae*, and directed mutants were obtained by selective plating. Correct integration of the antibiotic resistance cassette into the target gene was validated by PCR. Gene deletion mutants were crossed back to selective plating. Construction of pneumococcal mutants was achieved using allelic exchange of the target gene with an antibiotic resistance marker as described previously (10).

**Plasmid construction.** All plasmids used in this study are listed in Table 2. To obtain the plasmids for complementation of the CO$_2$-dependent growth defect of the *Δpca* strains, the *pca* gene of *S. pneumoniae* TIGR4 and the *ecu* gene (ECDH10B_106) of *E. coli* DH5α were PCR amplified with the PfBISP1CA L/
The PCA gene is required for pneumococcal growth under CO₂-poor conditions. To determine the importance of the pca gene for pneumococcal growth, pca deletion mutants (∆pca) were constructed from three S. pneumoniae strains, i.e., R6 (∆strp0026), D39 (∆spd_0030), and TIGR4 (∆ssp_0024). All ∆pca strains were able to grow normally on BA plates and Trypticase soy broth (TSB) agar plates supplemented with catalase (Trypticase soy agar [TSA]) under ambient air enriched with 5% CO₂ (data not shown). In vitro growth rates in...
5%-CO2-enriched GM17 broth medium were similar for the \( \Delta pca \) and wild-type strains, with cultures reaching an OD\(_{620} \) of 0.3 or more (Fig. 1A, left panel). In GM17 broth medium that was exposed to ambient air, the wild-type strains were also able to reach a high OD\(_{620} \). In contrast, growth of all \( \Delta pca \) strains under these CO\(_2\)-poor (0.038%) growth conditions was attenuated, and cultures did not reach an OD\(_{620} \) above 0.1 (Fig. 1A, right panel). Growth of the \( \Delta pca \) strains under CO\(_2\)-poor conditions was also impaired on TSA plates and reduced on BA plates (data not shown).

To exclude polar effects due to disruption of the \( pca \) gene, we provided the \( pca \) gene in trans on the pUO3 plasmid behind a nisin-inducible promoter. Induction of \( pca \) gene expression by the addition of nisin restored growth of the nisin-responsive \( \Delta pca \) strain harboring either pNG8048E (empty vector), pUO1 (ecca), or pUO3 (pca) in CO\(_2\)-poor GM17 broth medium without (−nis) and with (+nis) 20 ng/ml nisin. Growth of the pneumococcal cultures was monitored by recording the OD\(_{620} \). All curves in the graph present the averages of the results of three independent growth experiments.

**PCA has carbonic anhydrase activity.** The PCA enzyme was further characterized with enzymatic activity and inhibition assays. To facilitate the measurement of PCA enzymatic activity, PCA was overproduced as a GST fusion protein in \( E. coli \). Since no endogenous \( E. coli \) CA activity was detected in the lysates of control cells expressing only the GST protein, the CA activity in \( E. coli \) cells expressing GST-PCA can be fully ascribed to the presence of the recombinant protein (data not shown). The affinity-purified recombinant GST-PCA protein catalyzed the conversion of CO\(_2\) to HCO\(_3^-\) at pH 8.4, whereas the enzymatic activity was almost completely abrogated at pH 7.5 (Fig. 2A). Sulfonamides such as AZA and EZA are broad-range CA inhibitors that are active against most CAs (39), including the homologous Rv1284 CA in *Mycobacterium tuberculosis* (29). Interestingly, the presence of 100 \( \mu M \) AZA or 100 \( \mu M \) EZA did not reduce the CA activity of recombinant GST-PCA, whereas that of hCAII was completely inhibited (Fig. 2B).
CO2-poor GM17 broth medium, we complemented pneumo-
contain an essential HCO3
ways for nucleic acids, fatty acids, and several amino acids all

2B). Since both compounds also did not induce CO2 depen-
dence in S. pneumoniae wild-type strains (data not shown),
these sulfonamides are unlikely to have high affinity for PCA.

PCA is linked to UFA biosynthesis. The biosynthesis path-
ways for nucleic acids, fatty acids, and several amino acids all
contain an essential HCO3
various metabolic intermediates (i.e., adenine, uracil, arginine,

FIG. 3. Bicarbonate and oleic acid revert the CO2 dependence of Δpca strains. (A) Growth of the S. pneumoniae TIGR4Δpca strain in CO2-poor GM17 broth medium supplemented with NaHCO3 (10 mM), adenine (200 μg/ml), uracil (200 μg/ml), arginine (200 μg/ml), aspartic acid (200 μg/ml), palmitic acid (0.01 mM in 0.1% Tween 40), or oleic acid (0.01 mM in 0.1% Tween 40). (B) Growth of the S. pneumoniae TIGR4Δpca strain in CO2-poor GM17 broth medium with 0.1% Tween 20, Tween 40, or Tween 80. The growth of all pneumococcal broth cultures was monitored by recording the OD620. All curves in the graph present the averages of the results of three independent growth experiments.

Because supplementation with SFAs could not reverse the
CO2-dependent growth inhibition of the Δpca strains, CO2
fixation by PCA appears to be essential when insufficient UFAs
are available in the growth medium. The synthesis of UFAs
and SFAs in S. pneumoniae occurs essentially by the same
pathway (27). The dependency of the Δpca strains on UFA
supplementation for growth under CO2-poor conditions there-
fore suggests that under this condition UFAs are more readily
depleted. Recently, it was reported that the reactive oxygen
species (ROS) scavenger salicylate increased the unsaturation
index of bacterial-membrane fatty-acyl chains under aerobic
(thus CO2-poor) growth conditions by protecting UFAs
against endogenous oxidative stress (31). In line with this ob-
ervation, cultures of the S. pneumoniae R6Δpca and
TIGR4Δpca strains grown under CO2-poor conditions reached
an almost-2-fold-higher optical density when supplemented
with salicylate (Fig. 4A). Neutralization of endogenous H2O2,
which also plays an important role in lipid peroxidation (38),
through the addition of high concentrations of catalase re-
stored growth of the S. pneumoniae R6Δpca and TIGR4Δpca
strains to an almost-3-fold-higher optical density (Fig. 4B).
Despite the involvement of pyruvate oxidase (spxB) in endog-
ous H2O2 production (41), disruption of the spxB gene in the
TIGR4Δpca and R6Δpca strains did not restore growth to the
same level as that in the catalase-complemented cultures (Fig.
4B). Moreover, the addition of catalase still promoted growth
of the S. pneumoniae TIGR4ΔspxBΔpca and R6ΔspxBΔpca
strains (Fig. 4B).

PCA is required for intracellular survival inside host cells.
Membrane fatty acids are essential for pneumococcal growth
and survival (26) and are important targets for host defense
mechanisms (38). Because our experiments suggest that PCA
activity and UFA biosynthesis are linked, we investigated the
specific contribution of PCA to pneumococcal host-pathogen
interactions. To identify PCA-mediated effects on the interac-
tion of S. pneumoniae with host cells, we assessed the ability of
the Δpca strains to adhere to, invade, and survive in different
cell lines. First, we studied the interaction of S. pneumoniae
with human pharyngeal epithelial Detroit 562 cells, which are
representative of the host cells encountered by S. pneumoniae
during colonization of human upper airways. Disruption of the
pca gene in the unencapsulated (Δcps) derivative of S. pneumoniae
TIGR4 strain did not lead to decreased adherence to
(Fig. 5A) or invasion of (Fig. 5C) these epithelial cells.
However, at 1 h after pneumococcal invasion of the host cells,
we observed a statistically significant 1.3-fold reduction in in-
cellular survival of the TIGR4ΔcpsΔpca strain in Detroit
562 cells (Fig. 5E). Next, we examined the role of PCA during
interaction of S. pneumoniae with the HBMEC line. Endothel-
ial cells are the main component of the blood-brain barrier,
and penetration of this barrier by pathogens can lead to men-
ingitis. Adherence to HBMEC was not significantly different between the TIGR4Δcps and TIGR4ΔcpsΔpca strains (Fig. 5A). In contrast, the number of viable intracellular bacteria that could be recovered from HBMECs directly after phagocytosis was reduced 7-fold for the TIGR4ΔcpsΔpca strain compared to that of the TIGR4Δcps strain (Fig. 5C). Interestingly, at 2 and 4 h after invasion, the relative decreases in the numbers of intracellular bacteria were equal for the two strains (Fig. 5F). Finally, we investigated the role of PCA during interaction of S. pneumoniae with mouse J774 macrophage cells, which are primary immune cells important for clearance of bacterial infections. Exposure of J774 cells to TIGR4Δcps induced morphological and phenotypical changes in J774 cells, such as surface detachment and cell lysis, making readout unreliable and leading to nonreproducible results.

**FIG. 4.** Scavengers for endogenous ROS delay the CO2-dependent growth defect of Δpca strains. (A) Growth of the R6 and TIGR4 S. pneumoniae Δpca strains in CO2-poor GM17 broth medium without (+sal) or with 5 mM (−sal) sodium salicylate. (B) Growth of the S. pneumoniae R6 and TIGR4Δpca and ΔpcaΔspxB strains in CO2-poor GM17 broth medium without (−cat) or with (+cat) 10,000 U/ml of catalase. The growth of all pneumococcal broth cultures was monitored by recording the \( \text{OD}_{620} \). All curves in the graphs present the results of a single experiment that are characteristic of those for three independent growth experiments.

**DISCUSSION**

The respiratory tract pathogen S. pneumoniae needs to adapt to the various conditions it encounters during transmission, colonization, and disease. Currently, relatively little is known about the genetic and metabolic factors that contribute to an adequate response of this bacterium to changes in CO2 availability. In this study, we showed that the putative carbonic anhydrase in S. pneumoniae has an important role for growth in CO2-poor conditions.

Our experiments clearly showed that the pca gene encodes a functionally active carbonic anhydrase. All the Δpca strains were growth deficient in CO2-poor conditions but could be complemented by the addition of HCO\(_3^-\), the expected end product of PCA enzymatic activity. In addition, growth of the Δpca strains could be restored by in trans expression of the...
Finally, recombinant GST-PCA was able to catalyze the conversion of CO$_2$ to HCO$_3^-$, which is interestingly, PCA did not appear to be active at the physiological pH of 7.5. This is not unusual for β-CAs and has been observed for ECCA and the _H. influenzae_ CA (HICA). Most likely, this pH-dependent behavior is linked to the pH-dependent coordination of Zn$^{2+}$ in the active site (13). Furthermore, both ECCA and HICA appear to have an alternative bicarbonate binding site that renders the enzyme inactive at physiological pH when sufficient substrate is present (13). Although PCA appears to miss essential amino acids that form the alternative bicarbonate binding site, it is also not unlikely that differences exist between its CA activity in enzymatic assays and in physiological conditions. Another striking characteristic of PCA is its lack of affinity for broad-range carbonic anhydrase inhibitors. This indicates that this enzyme is deviant from other well-characterized CAs, which is not surprising, as there is huge variation among the different CAs, and CA inhibitors were often developed against unrelated hCAs. In fact, differences between PCA and hCAs could benefit the therapeutic potential of PCA inhibitors.

**TABLE 3. Characteristics of CO$_2$-dependent carriage isolates**

<table>
<thead>
<tr>
<th>Strain$^a$</th>
<th>Transformation (CFU/ml) with DNA from:</th>
<th>Growth on or in indicated plate or broth$^b$</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>R6</td>
<td>R6 Δpca</td>
<td>BA plates</td>
<td>TSA plates</td>
<td>GM17 broth</td>
</tr>
<tr>
<td>H23</td>
<td>19,800</td>
<td>14,900</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>H26</td>
<td>67</td>
<td>67</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

$^a$ Both strains carry the pca gene.

$^b$ No. of colonies growing on BA plates under CO$_2$-poor conditions.

$^c$ +, growth; –, no growth.

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**FIG. 5.** PCA is required for invasion and intracellular survival in host cells. (A and B) *In vitro* adherence of the TIGR4 Δcps Δpca strain to Detroit 562 cells and HBMECs (A) and binding of the D39 Δcps Δpca strain by J774 cells (B). The relative adherence and binding efficiencies were correlated to those of the TIGR4 Δcps and D39 Δcps strains, respectively. (C and D) Invasive properties of the TIGR4 Δcps Δpca strain toward Detroit 562 cells and HBMECs (C) and uptake of the D39 Δcps Δpca strain by J774 cells (D). The relative invasion and uptake efficiencies were correlated to the number of viable intracellular cells of the TIGR4 Δcps and D39 Δcps strains, respectively. (E and F) Intracellular survival kinetics of the TIGR4 Δcps and TIGR4 Δcps Δpca strains in Detroit 562 cells (E) and HBMECs (F). (G) Phagocytic killing of the D39 Δcps and D39 Δcps Δpca strains in J774 cells. Intracellular survival and phagocytic killing were correlated to viable-bacteria counts at time zero, *, statistically significant differences ($P < 0.05$).
quired for the carboxylation of acetyl coenzyme A (acetyl-CoA) by acetyl-CoA carboxylase to form malonyl-CoA, which is the first committed step of FA biosynthesis (15). We did not observe a stimulating effect of any of the other tested metabolic intermediates on the growth of the S. pneumoniae Δpca strains in CO2-poor GM17 broth medium. This implies that GM17 medium contains limiting amounts of UFAs but sufficient levels of the other metabolites to support growth. Based on the UFA supplementation experiments and previous observations of other microorganisms (1, 5), we can predict that other carboxylation reactions, e.g., those involved in biosynthesis of some amino acids, pyrimidines, and purines, also depend on PCA activity when CO2 levels are low. Still, we feel that support of UFA biosynthesis is one of the most relevant aspects of PCA function. Although S. pneumoniae is able to tolerate low levels of membrane SFAs, insufficient UFAs lead to decreased cell viability (2). In ambient-air conditions, both environmental and cellular UFAs are prone to oxidation and can be replaced only by the PCA-supported biosynthesis of UFAs. In addition, endogenous production of ROS by S. pneumoniae itself leads to increased cellular UFA peroxidation (31). Due to the transient phenotype of the pca mutation, it was not possible to perform a straightforward experiment to directly link the disruption of the pca gene to an alteration in the membrane FA composition or increased ROS sensitivity. In the absence of CO2, the Δpca strains do not grow, whereas in the presence of CO2, the Δpca and wild-type strains are phenotypically identical. In analogy with studies of S. pneumoniae UFA auxotrophs (27), we did attempt to complement cultures of the Δpca strains in CO2-poor conditions with UFAs to restore growth and allow characterization of membrane FAs. However, supplementation of cultures of the pneumococcal wild-type and Δpca strains with UFAs completely repressed expression of the FA biosynthesis gene cluster (our unpublished data), which inevitably results in a membrane that is predominantly composed of exogenous FAs (9).

It is tempting to speculate about the role of PCA in neutralizing the detrimental effect of pneumococcal SpxB activity. In ambient air, SpxB produces H2O2, acetyl-phosphate, and CO2. Production of H2O2 leads to UFA peroxidation (31), whereas acetyl-phosphate can readily be converted to acetyl-CoA by phosphate acetyl-transferase to support de novo FA biosynthesis. PCA then acts to convert CO2 to HCO3−, allowing carboxylation of acetyl-CoA to form malonyl-CoA. Currently, this hypothesis is not supported by our own observations, as catalase improved growth of both the S. pneumoniae Δpca and ΔpcaΔspxB strain cultures. However, the interconnection between SpxB activity and FA biosynthesis is still poorly understood and might involve different metabolic and regulatory pathways (31, 44). Alternatively, this suggests that other sources of endogenous oxidative stress, such as the Fenton reaction (31) or lactate oxidase activity (44), have a profound impact on the growth arrest of the Δpca strains in CO2-poor conditions as well.

The role of PCA in the de novo biosynthesis of UFAs and, possibly, other metabolites could also explain the decreased viability of the S. pneumoniae Δpca strains after invasion of endothelial cells and uptake by macrophages. During phagocytosis, and possibly endocytosis (33), a substantial portion of the intracellular bacteria is sorted to the host-cell lysosome. The low pH of this compartment reduces HCO3− availability, and the production of ROS leads to peroxidation of bacterial membrane UFAs (38) and nucleic acids (37). Interestingly, the effect of pca disruption on S. pneumoniae invasion and intracellular survival inside Detroit 562 pharyngeal epithelial cells was not as pronounced as in the two other cell types. Whether this difference reflects on the different routes for pneumococcal invasion of Detroit 562 cells by interaction with the polymeric immunoglobulin receptor (pIgR) (49) and HBMECs by interaction with the platelet-activating factor receptor (PAF) (34) remains to be studied. A role for microbial carbonic anhydrases inside host cells was earlier suggested for a Salmonella enterica serovar Typhimurium CA (mig-5), which was expressed after uptake in macrophages and a mutant of which had a marked decrease in spleen colonization of mice (46). In contrast to findings for Salmonella CA mutants, we were not able to link PCA with virulence in animal models of bacteremia. However, this observation is in line with the outcome of a previous study showing that mice deficient in the NADPH oxidase subunit gp91, which is essential for lysosomal ROS production, were as sensitive to pneumococcal infection as wild-type mice (36). Furthermore, it is known that pneumococcal capsular polysaccharides prevent recognition and uptake of the bacterium by host immune cells, and once S. pneumoniae remains extracellular during infection of the blood, it might utilize serum HCO3− and FAs (9). Possibly, the role of PCA in pneumococcal disease is more pronounced in animal models of disease in which the bacterium needs to traverse the boundaries of epithelial and endothelial cells for dissemination from the respiratory tract to the blood and cerebrospinal fluid. Finally, the role of PCA in S. pneumoniae can be projected onto CAs in other (respiratory tract) pathogens. Although CAs are ubiquitous enzymes in many microorganisms, most studies have investigated the role of CAs that are exposed to the surface or periplasm, have species-specific functions, or do not belong to the class of β-CAs (39). Here, we show that cytosolic β-CAs related to PCA are involved in FA biosynthesis and may offer novel opportunities for the design of broad-range therapies. Furthermore, PCA is probably only one of the factors that contribute to the adaptation of S. pneumoniae to CO2-poor conditions, which might be relevant for pneumococcal transmission in environmental ambient air. Detailed examination of the metabolic pathways that depend on PCA-mediated CO2 fixation and the identification of the genetic basis for the CO2 dependence observed in approximately 8% of all circulating pneumococcal isolates is expected to lead to novel insights into the way respiratory pathogens adapt to the CO2- and HCO3−-poor environments they encounter during transmission, colonization, and disease.

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