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Carbonic Anhydrase Is Essential for *Streptococcus pneumoniae* Growth in Environmental Ambient Air▽†

Peter Burghout,[1] Lorelei E. Cron,[1] Henrik Gradstedt,[2] Beatriz Quintero,[1,3] Elles Simonetti,[1] Jetta J. E. Bijlsma,[2] Hester J. Bootsma,[1] and Peter W. M. Hermans[1]*

Laboratory of Pediatric Infectious Diseases, Radboud University Nijmegen Medical Centre, Nijmegen, Netherlands[1]; Department of Medical Microbiology, University Medical Centre Groningen, and University of Groningen, Groningen, Netherlands[2]; and Department of Microbiology and Parasitology, Faculty of Medicine, University of Los Andes, Merida, Venezuela[3]

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The respiratory tract pathogen *Streptococcus pneumoniae* needs to adapt to the different levels of carbon dioxide (CO_2) it encounters during transmission, colonization, and infection. Since CO_2 is important for various cellular processes, factors that allow optimal CO_2 sequestering 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 *in vitro* growth of *S. pneumoniae* under the CO_2-poor conditions found in environmental ambient air. Enzymatic analysis showed that PCA catalyzes the reversible hydration of CO_2 to bicarbonate (HCO_3^-), an essential step to prevent the cellular release of CO_2. The addition of unsaturated fatty acids (UFAs) reversed the CO_2-dependent *in vitro* growth inhibition of *S. pneumoniae* strains lacking the *pca* gene (Δ*pca*), indicating that PCA-mediated CO_2 fixation is at least associated with HCO_3^--dependent *de novo* biosynthesis of UFAs. Besides being necessary for growth in environmental ambient conditions, PCA-mediated CO_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 *S. pneumoniae* with Detroit 562 pharyngeal epithelial cells. Finally, the highly conserved *pca* gene was found to be invariably present in both CO_2-independent and naturally circulating CO_2-dependent strains, suggesting a conserved essential role for PCA and PCA-mediated CO_2 fixation pathways for pneumococcal growth and survival.

The Gram-positive bacterium *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 capsular 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.

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, *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 *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 *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 fixated. Carbonic anhydrases

* Corresponding author. Mailing address: Laboratory of Pediatric Infectious Diseases, Radboud University Nijmegen Medical Centre, P.O. Box 9101 (Internal mail 224), 6500 HB Nijmegen, Netherlands. Phone: 31-24-3666406. Fax: 31-24-3666352. E-mail: p.hermans@cukz.umcn.nl.

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TABLE 1. Bacterial strains used in this study

Species and strain	Relevant characteristics	Reference
S. pneumoniae		
R6	Wild-type strain, unencapsulated	19
D39	Wild-type strain, serotype 2	25
TIGR4	Wild-type strain, serotype 4	45
R6Δ*pca*	Δspr0026 Sp^r	This study
D39Δ*pca*	ΔSPD_0030 Sp^r	This study
TIGR4Δ*pca*	ΔSP_0024 Sp^r	This study
R6*bga*::*nisRK*	Nisin-responsive R6 strain; spr0565::nisRK Tmp^r	23
R6*bga*::*nisRK*Δ*pca*	Nisin-responsive R6 strain; spr0565::nisRK Δspr0026 Tmp^r Sp^r	This study
R6Δ*pca*Δ*spxB*	Δspr0026 Δspr0642 Sp^r Km^r	This study
TIGR4Δ*pca*Δ*spxB*	ΔSP_0024 ΔSP_0730 Sp^r Km^r	This study
TIGR4Δ*cps*	Unencapsulated TIGR4 strain; ΔSP_0343-0365 Km^r	11
TIGR4Δ*cps*Δ*pca*	Unencapsulated TIGR4 strain; ΔSP_0343-0365 ΔSP_0024 Km^r Sp^r	This study
D39Δ*cps*	Unencapsulated D39 strain; ΔSPD_0312-0333 Km^r	11
D39Δ*cps*Δ*pca*	Unencapsulated D39 strain; ΔSPD_0312-0333 ΔSPD_0030 Km^r Sp^r	This study
H23	CO_2-dependent carriage isolate	Laboratory collection
H26	CO_2-dependent carriage isolate	Laboratory collection
E. coli		
DH5α	Cloning strain	17
BL21	Expression strain	Novagen
L. lactis		
NZ9000	Cloning strain	24

Sp^r, spectinomycin resistant; Tmp^r, trimethoprim resistant; Km^r, kanamycin resistant.

(CAs; EC 4.2.1.1) are enzymes that catalyze the reversible reaction $CO_2 + H_2O \leftrightarrow HCO_3^- + H^+$. 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 (Biotrading). 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 (Δ*pca*), 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 at 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; chloroamphenicol, 2.5 μg/ml for *S. pneumoniae* and 5 μg/ml for *L. lactis*; adenine, 5 mg/ml in 0.05 M HCl; uracil, 2 mg/ml in 1% sodium carbonate (Na_2CO_3); arginine, 20 mg/ml; aspartic acid, 20 mg/ml (pH 7); palmitic acid or oleic acid, 200 mM 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 Qiaprep 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, AmpliTaq DNA polymerase (Applied Biosystems) was applied. The primers (Biolegio, 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 pR410 or pR412 plasmid (Table 2), respectively, between the two 500-bp flanking sequences surrounding the target gene. 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 the wild-type strain, using chromosomal DNA of the mutant strains as the donor during transformation. Since the flanking sequences of all the target genes used in this study were homologous in the R6, D39, and TIGR4 strains, gene deletions were introduced into D39 and TIGR4 by transformation with the PCR-amplified gene deletions and 500-bp flanking sequences of the R6 mutant derivatives.

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 Δ*pca* strains, the *pca* gene of *S. pneumoniae* TIGR4 and the *ecca* gene (ECDH10B_106) of *E. coli* DH5α were PCR amplified with the PBNISPCA_L/

TABLE 2. Plasmids used in this study

Plasmid	Relevant characteristics[a]	Reference
pR410	Donor for kanamycin resistance cassette	43
pR412	Donor for spectinomycin resistance cassette	28
pCR2.1	Cloning vector; Ap^r Km^r	Invitrogen
pGEX-1N	Expression vector with N-terminal GST tag; Ap^r	Novagen
pNG8048E	Expression vector with nisin-inducible promoter; Ca^r	23
pWA1	pCR2.1 with *pca* gene, BamHI site; Ap^r Km^r	This study
pWA4	pGEX-1N with *gst-pca* construct; Ap^r	This study
pCR2.1-PCA_L	pCR2.1 with *pca* gene, BsaI site; Ap^r Km^r	This study
pCR2.1-ECCA	pCR2.1 with *ecca* gene, BsaI site; Ap^r Km^r	This study
pUO1	pNG8048 with *ecca* gene behind nisin-inducible promoter; Ca^r	This study
pUO3	pNG8048 with *pca* gene behind nisin-inducible promoter; Ca^r	This study

[a] Ap^r, ampicillin resistant; Km^r, kanamycin resistant; Ca^r, chloroamphenicol resistant.

PBNISPCA_R and PB_NISECCA_L/PB_NISECCA_R primer pairs, respectively. The PCR products were cloned into the pCR2.1 cloning vector of a TA cloning kit (Invitrogen) to obtain pCR2.1-PCA_L and pCR2.1-ECCA, respectively. In the next step, the genes were excised by BsaI/EcoRI digestion and ligated to the NcoI/EcoRI-digested pNG8048 plasmid to obtain pUO3 and pUO1, respectively. To obtain the plasmid for overproduction of GST-PCA, the *pca* gene of *S. pneumoniae* TIGR4 was PCR amplified with the PBPCA_S/PBPCA_E primer pair and cloned into pCR2.1 to obtain pWA1. In the next step, the *pca* gene was excised by BamHI/EcoRI digestion and subcloned behind the GST gene in a BamHI/EcoRI-digested pGEX-1N vector to obtain pWA4. Cloning of the pCR2.1 and pGEX-1N plasmids was performed in *E. coli* DH5α, and cloning of the pNG8048E plasmids was performed in *L. lactis* NZ9000. The nucleotide sequences of the PCR products in the pCR2.1 plasmid were confirmed by sequencing.

Production and purification of recombinant GST-PCA. For GST-PCA production, an overnight culture of *E. coli* BL21 (pWA4) was diluted 50-fold in prewarmed (37°C) 2× LB supplemented with 0.5% glucose. At an optical density at 600 nm (OD_{600}) of 0.6 to 0.8, 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) was added, and cultures were shifted to room temperature. After 4 h, cells were placed on ice, pelleted by centrifugation, resuspended in ice-cold lysis buffer (50 mM Tris-HCl [pH 7.5], 300 mM NaCl, 0.5 mM dithiothreitol [DTT], 1.5 mM $MgCl_2$, 0.2 mM EDTA, 1% Triton X-100) with 1× protease inhibitor mixture (Complete Mini; Roche Applied Science) to a cell density equivalent to an OD_{600} of 100, and lysed by sonication. Insoluble debris in the lysate was removed by centrifugation at 16,000 × *g* for 10 min at 4°C, and the supernatant was incubated overnight with prewashed (1× PBS) glutathione Sepharose 4 Fast Flow beads (GE Healthcare) at 4°C. Nonspecifically bound proteins were removed by washing the beads three times with lysis buffer for 15 min at 4°C. GST-PCA was eluted with elution buffer (50 mM Tris-HCl [pH 8.0], 300 mM NaCl, 10 mM glutathione, and 0.5 mM DTT). The eluate was dialyzed against 50 mM Tris, pH 7.5. The protein concentration in the GST-PCA solution was determined with a bicinchoninic acid (BCA) protein assay kit (Pierce).

Carbonic anhydrase activity assay. The activity of CAs was determined by the changing-pH/dye indicator method (22) on an RX.2000 rapid-mixing stopped-flow unit (Applied Photophysics, United Kingdom). Briefly, enzyme samples were diluted in reaction buffer at pH 7.5 (50 mM HEPES [pH 7.5], 200 mM phenol red, and 200 mM Na_2SO_4) or at pH 8.4 (50 mM TAPS [pH 8.4], 200 mM m-cresol purple, and 200 mM Na_2SO_4), and the reaction was initiated by the addition of an equivalent amount of CO_2-saturated water. The subsequent restoration of CO_2/HCO_3^- balance was monitored by the color conversion of the pH-sensitive dye indicators at 558 nm (pH 7.5) or 578 nm (pH 8.4). All reactions were performed at 25°C. The CA activities of GST-PCA and human CA II ([hCAII] Sigma) were measured at final concentrations of 100 μg/ml and 0.5 μg/ml, respectively. When appropriate, 50 mM Tris-HCl (pH 7.5) and dimethyl sulfoxide (DMSO) were included as nonenzymatic controls. The stock solutions for CA inhibition studies were 100 mM acetazolamide ([AZA] Sigma) and 100 mM ethoxyzolamide ([EZA] Sigma) in DMSO.

Cell lines, culture conditions, and host-pathogen studies. The human pharyngeal epithelial cell line Detroit 562 (CCL-138; ATCC) was routinely grown in RPMI 1640 medium without phenol red (Invitrogen, Netherlands) supplemented with 1 mM sodium pyruvate and 10% fetal calf serum (FCS). The human brain microvascular endothelial cell (HBMEC) line was cultivated in RPMI 1640 medium supplemented with 10% FCS, 10% Nu-Serum, 2 mM L-glutamine, 1 mM sodium pyruvate, 1% minimal essential medium (MEM)-vitamins, and 1% nonessential amino acids (42). Prior to infection, HBMEC monolayers were incubated for 1 h in culture medium with 10 ng/ml of tumor necrosis factor alpha (TNF-α). The murine macrophage-like cell line J774 (TIB 67; ATCC) was cultured in Dulbecco's modified Eagle's medium (DMEM) GlutaMAX-I (Invitrogen, Netherlands) with 10% FCS. All cells were cultured in a 5%-CO_2 incubator at 37°C.

Pneumococcal adherence, invasion, and intracellular survival studies were performed essentially as described previously (7, 11, 16). Briefly, monolayers of J774, Detroit 562, or HBMECs were infected with bacteria in 5%-CO_2-enriched culture medium with only 1% FCS (infection medium). Subsequently, the pneumococci were allowed to adhere to the cells for 0.5 h, 1 h, or 2 h, respectively, and nonadherent bacteria were removed by washing. To quantify adherence, host cells were detached from the wells and lysed with 0.025% Triton X-100 or 1% saponin and trypsin-EDTA (0.05%-0.02%). To determine the level of invasion into the host cells, extracellular *S. pneumoniae* cells were killed by a 1-h incubation with 1 ml 5%-CO_2-enriched infection medium supplemented with gentamicin (200 μg/ml) and penicillin G (10 μg/ml) before cells were lysed. To examine intracellular survival, cells were infected and treated with gentamicin and penicillin G as described above, after which cells were washed once and fresh 5%-CO_2-enriched medium containing gentamicin (13.34 μg/ml) and penicillin G (0.67 μg/ml) (1/15 of beginning antibiotic concentration) was added to each well for prolonged incubation. For all *in vitro* cell culture studies, the pneumococcal wild-type and mutant strains grew comparably in infection medium alone. Results were corrected mathematically to account for small differences in count in the initial inoculum.

***In vivo* colonization and bacteremia experiments.** Bacteremia and nasopharyngeal colonization experiments with mice were conducted with 9-week-old female outbred CD-1 mice (Harlan, Horst, Netherlands) as described recently (18). Briefly, for the colonization experiments, 1×10^6 CFU in 10 μl of PBS were administered to the nostrils of groups of five mice for each strain, and bacteria were recovered from the nasopharynx by flushing the nose with 2 ml of sterile PBS at 96 h. Bacteremia experiments were performed twice with groups of at least five mice for each strain. Mice were infected intravenously in the tail vein with 1×10^6 CFU in 100 μl of PBS, and bacteria were recovered from the blood by retro-orbital puncture. For mice in which no bacteria were found, a lower limit of detection (22 CFU/ml) was used. Results were corrected mathematically to account for small differences in bacterial count in the initial inoculum. All experiments were performed with the approval of the Animal Experimentation Committee (DEC) of the Radboud University Nijmegen Medical Centre.

***In silico* analysis.** The subcellular location of PCA enzyme was predicted by various online prediction servers, such as PSORTb (http://www.psort.org) and TMHMM (http://www.cbs.dtu.dk/services/TMHMM). Conservation of the *pca* gene and PCA protein was performed by the genomic BLAST service on the website of the National Center for Biotechnology Information (NCBI) of the U.S. National Library of Medicine (http://www.ncbi.nlm.nih.gov/).

Statistical analysis. For *in vitro* host-pathogen studies, data were analyzed using an unpaired Student's *t* test, with *P* values of <0.05 considered significant. All statistical analyses were performed using GraphPad Prism version 4.0.

RESULTS

The *pca* gene is required for pneumococcal growth under CO_2-poor conditions. To determine the importance of the *pca* gene for pneumococcal growth, *pca* deletion mutants (Δ*pca*) were constructed from three *S. pneumoniae* strains, i.e., R6 (Δspr0026), D39 (ΔSPD_0030), and TIGR4 (ΔSP_0024). All Δ*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% CO_2 (data not shown). *In vitro* growth rates in

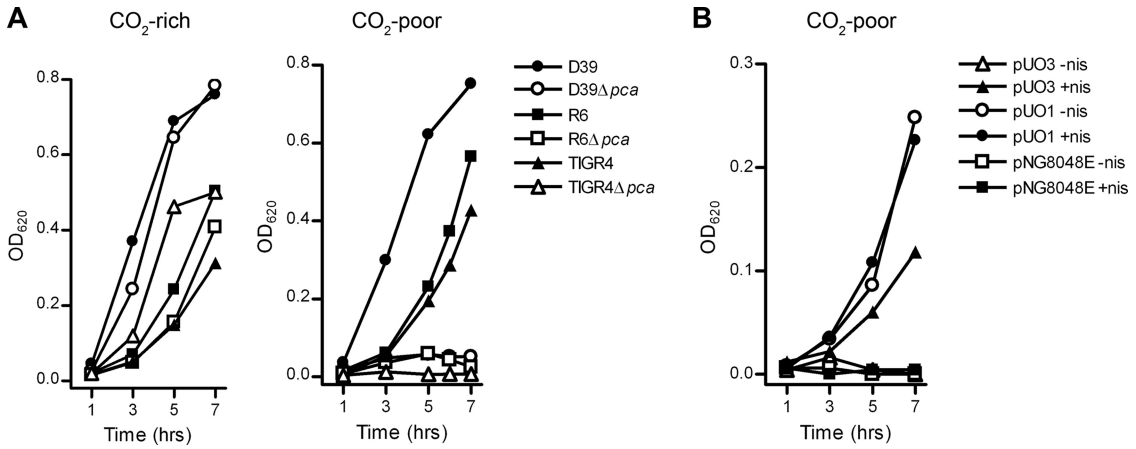

FIG. 1. Disruption of the *pca* gene in *S. pneumoniae* leads to CO_2-dependent growth inhibition. (A) Growth characteristics of the *S. pneumoniae* R6, D39, and TIGR4 wild-type and Δ*pca* strains in CO_2-rich and CO_2-poor GM17 broth medium. (B) Growth of the *S. pneumoniae* R6*bga*::*nisRK* Δ*pca* 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.

5%-CO_2-enriched GM17 broth medium were similar for the Δ*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 Δ*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 Δ*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 R6*bga*::*nisRK*Δ*pca* (pUO3) strain in CO_2-poor GM17 broth (Fig. 1B). Introduction of the pUO1 plasmid with the gene for *E. coli* carbonic anhydrase (ECCA) (12) into R6*bga*::*nisRK*Δ*pca* also reversed the CO_2 dependence of this strain (Fig. 1B). Interestingly, complementation by ECCA did not appear to require induction with nisin. Because pUO1 could not restore the CO_2 dependence of the R6Δ*pca* strain lacking the NisRK sensor for nisin (data not shown), it is likely that autoinduction of the NisRK two-component signal transduction system resulted in expression of small but sufficient amounts of ECCA.

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 μM AZA or 100 μM EZA did not reduce the CA activity of recombinant GST-PCA, whereas that of hCAII was completely inhibited (Fig.

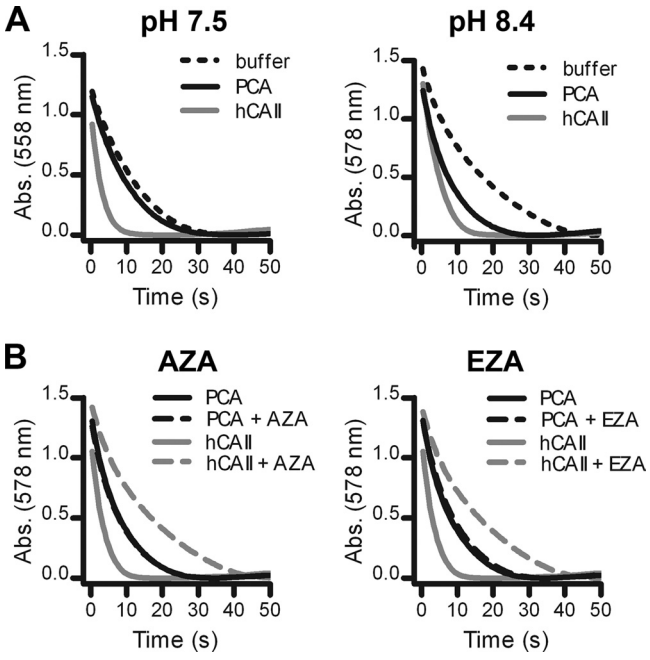

FIG. 2. Enzymatic activity and inhibition studies of recombinant GST-PCA. (A) The CA activity of GST-PCA (100 μg/ml) was measured by the changing-pH/dye indicator method at pH 7.5 and pH 8.4. (B) Inhibitory effect of the sulfonamides AZA (100 μM) and EZA (100 μM) on the CA activity of GST-PCA at pH 8.4. Under all conditions tested, hCAII (0.5 μg/ml) and nonenzymatic reactions were included as positive and negative controls, respectively. The curves for the nonenzymatic control reactions of the inhibition study overlapped with the curves for hCAII with an inhibitor and for the clarity of the graph were not displayed. All curves in the graphs present the averages of the results of three independent CA activity assays. Abs., absorbance.

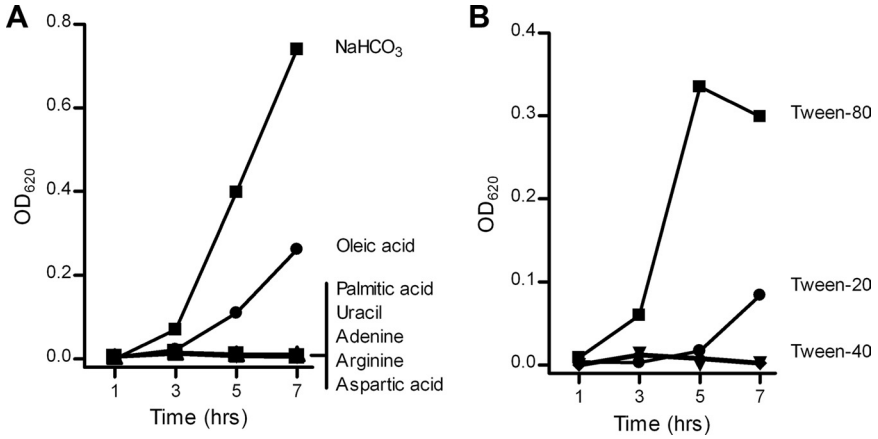

FIG. 3. Bicarbonate and oleic acid revert the CO_2 dependence of Δ*pca* strains. (A) Growth of the *S. pneumoniae* TIGR4Δ*pca* strain in CO_2-poor GM17 broth medium supplemented with $NaHCO_3$ (10 mM), adenine (200 μg/ml), uracil (200 μg/ml), arginine (200 μg/ml), aspartic acid (200 μg/ml), palmitic 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* TIGR4Δ*pca* strain in CO_2-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 OD_{620}. All curves in the graph present the averages of the results of three independent growth experiments.

2B). Since both compounds also did not induce CO_2 dependence 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 pathways for nucleic acids, fatty acids, and several amino acids all contain an essential HCO_3^--dependent carboxylation step that could potentially account for the observed growth defect of microbial CA mutants in CO_2-poor conditions (1). To investigate if one or more of these carboxylation steps are responsible for the growth inhibition of the *S. pneumoniae* Δ*pca* strains in CO_2-poor GM17 broth medium, we complemented pneumococcal cultures with sodium hydrogen carbonate ($NaHCO_3$) or various metabolic intermediates (i.e., adenine, uracil, arginine, aspartic acid, palmitic acid [in 0.1% Tween 40], or oleic acid [in 0.1% Tween 40]) (Fig. 3A). As predicted, $NaHCO_3$ fully reversed growth of the *S. pneumoniae* TIGR4Δ*pca* strain under CO_2-poor conditions. The unsaturated fatty acid (UFA) oleic acid was the only metabolic intermediate that could partially restore growth as well, although not to the same level as $NaHCO_3$. Other sources of UFAs, such as Tween 20 and Tween 80 (30), could also (partially) reverse the CO_2 dependence of the *S. pneumoniae* TIGR4Δ*pca* strain (Fig. 3B). In contrast, the saturated fatty acid (SFA) palmitic acid (Fig. 3A) or Tween 40 (Fig. 3B), which is a Tween derivative that is solely composed of SFA, was ineffective.

Because supplementation with SFAs could not reverse the CO_2-dependent growth inhibition of the Δ*pca* strains, CO_2 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 Δ*pca* strains on UFA supplementation for growth under CO_2-poor conditions therefore 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 CO_2-poor) growth conditions by protecting UFAs against endogenous oxidative stress (31). In line with this observation, cultures of the *S. pneumoniae* R6Δ*pca* and TIGR4Δ*pca* strains grown under CO_2-poor conditions reached an almost-2-fold-higher optical density when supplemented with salicylate (Fig. 4A). Neutralization of endogenous H_2O_2, which also plays an important role in lipid peroxidation (38), through the addition of high concentrations of catalase restored growth of the *S. pneumoniae* R6Δ*pca* and TIGR4Δ*pca* strains to an almost-3-fold-higher optical density (Fig. 4B). Despite the involvement of pyruvate oxidase (SpxB) in endogenous H_2O_2 production (41), disruption of the *spxB* gene in the TIGR4Δ*pca* and R6Δ*pca* 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* TIGR4Δ*spxB*Δ*pca* and R6Δ*spxB*Δ*pca* 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 interaction of *S. pneumoniae* with host cells, we assessed the ability of the Δ*pca* 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 (Δ*cps*) derivative of the *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 intracellular survival of the TIGR4Δ*cps*Δ*pca* strain in Detroit 562 cells (Fig. 5E). Next, we examined the role of PCA during interaction of *S. pneumoniae* with the HBMEC line. Endothelial cells are the main component of the blood-brain barrier, and penetration of this barrier by pathogens can lead to men-

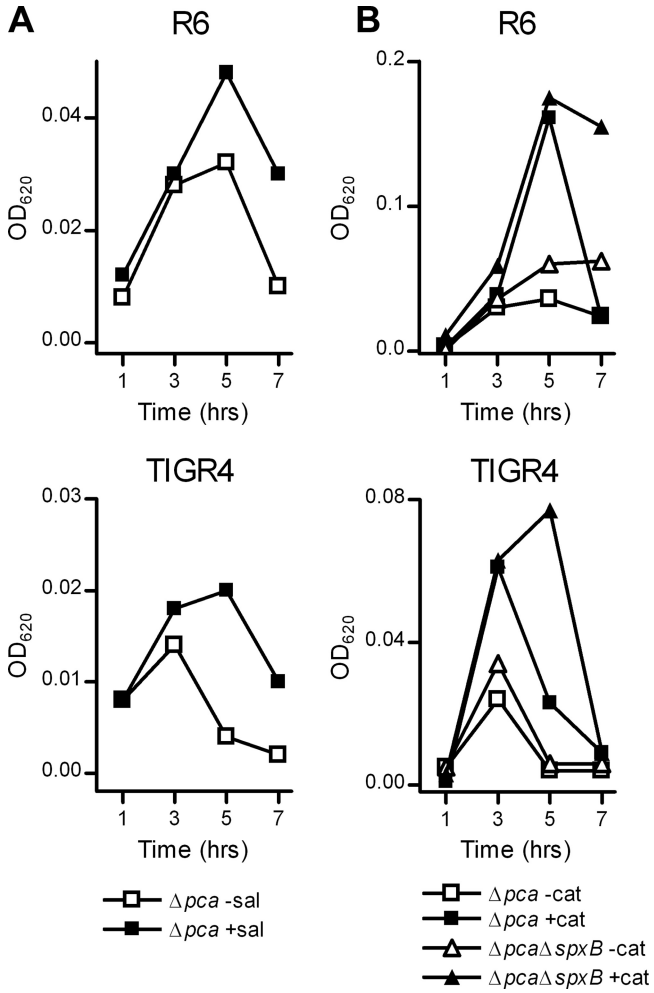

FIG. 4. Scavengers for endogenous ROS delay the CO_2-dependent growth defect of Δ*pca* strains. (A) Growth of the the R6 and TIGR4 *S. pneumoniae* Δ*pca* strains in CO_2-poor GM17 broth medium without (−sal) or with 5 mM (+sal) sodium salicylate. (B) Growth of the *S. pneumoniae* R6 and TIGR4Δ*pca* and Δ*pca*Δ*spxB* strains in CO_2-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 OD_{620}. All curves in the graphs present the results of a single experiment that are characteristic of those for three independent growth experiments.

ingitis. Adherence to HBMEC was not significantly different between the TIGR4Δ*cps* and TIGR4Δ*cps*Δ*pca* strains (Fig. 5A). In contrast, the number of viable intracellular bacteria that could be recovered from HBMECs directly after pneumococcal invasion was reduced 7-fold for the TIGR4Δ*cps*Δ*pca* strain compared to that of the TIGR4Δ*cps* strain (Fig. 5C). Interestingly, at 2 and 4 h after invasion, the relative decreases in the numbers of viable 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 TIGR4Δ*cps* induced morphological and phenotypical changes in J774 cells, such as surface detachment and cell lysis, making readout unreliable and leading to nonreproducible results. J774 interaction studies were therefore continued with the *S. pneumoniae* D39Δ*cps* and D39Δ*cps*Δ*pca* strains. Although disruption of the *pca* gene had no significant effect on the binding of *S. pneumoniae* by host immune cells (Fig. 5B), the number of viable intracellular bacteria directly after uptake by the macrophages was 2-fold lower for the D39Δ*cps*Δ*pca* strain than for the D39Δ*cps* strain (Fig. 5D). Moreover, temporal monitoring revealed that phagocytic killing of intracellular bacteria continued to be significantly faster for the D39Δ*cps*Δ*pca* strain than for the D39Δ*cps* strain (Fig. 5G). Despite the *in vitro* contribution of PCA to pneumococcal intracellular survival, no significant difference between the *S. pneumoniae* TIGR4 wild-type and TIGR4Δ*pca* strains was observed in mouse models of pneumococcal nasopharyngeal carriage and bacteremia (see Fig. S1 in the supplemental material).

The *pca* gene is present in CO_2-dependent circulating strains. The *pca* gene appears to be a highly conserved gene, which is present in all 11 complete and 18 draft *S. pneumoniae* genomes that are currently available in the public databases. Still, about 8% of all *S. pneumoniae* isolates from various sources have been reported as CO_2 dependent (3). To exclude the possibility that the *pca* gene is absent in these circulating strains, we investigated whether the CO_2 dependence of these isolates is related to the absence of a functional *pca* gene. Two out of 126 carriage strains (H23 and H26) isolated from healthy Venezuelan children (our unpublished data) did not grow on BA and TSA plates unless the environment was enriched with 5% CO_2. PCR analysis indicated that the *pca* gene was present in both CO_2-dependent strains (Table 3), and genetic transformation of these strains with chromosomal DNA from both the *S. pneumoniae* R6 wild-type and R6Δ*pca* strains resulted in CO_2-independent colonies (Table 3). These results suggest that the observed CO_2 dependence of the H23 and H26 strains is associated with a genetic defect or a missing gene other than *pca*. In addition, further phenotypical characterization of these strains showed that their CO_2 dependence was different from that of the *S. pneumoniae* Δ*pca* strains used in this study. Although both strains were completely CO_2 dependent for growth on BA plates, the H23 strains reached high optical densities in CO_2-poor GM17 broth medium. In contrast, the H26 strain did not grow at all in CO_2-poor GM17 broth medium, not even when it was supplemented with UFA (Tween 80) (Table 3).

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 CO_2 availability. In this study, we showed that the putative carbonic anhydrase in *S. pneumoniae* has an important role for growth in CO_2-poor conditions.

Our experiments clearly showed that the *pca* gene encodes a functionally active carbonic anhydrase. All the Δ*pca* strains were growth deficient in CO_2-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 Δ*pca* strains could be restored by in *trans* expression of the

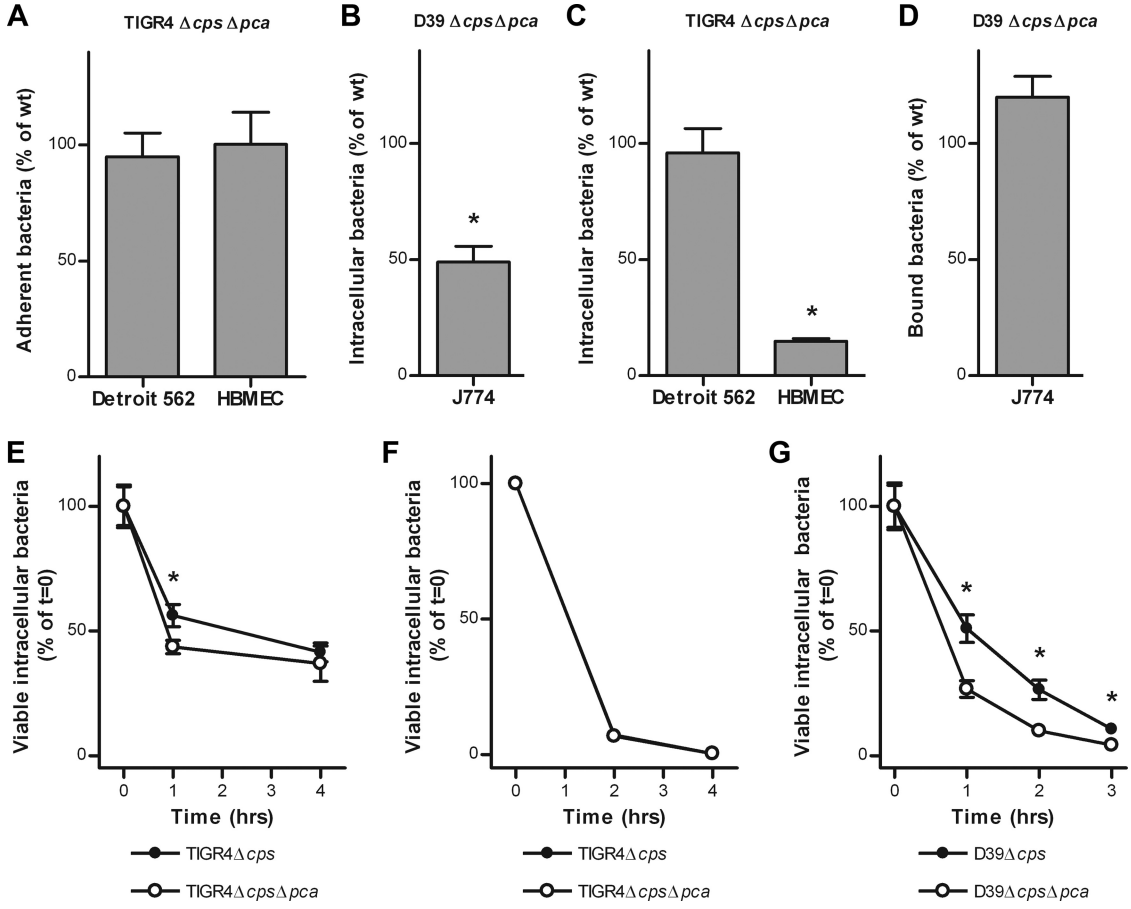

FIG. 5. PCA is required for invasion and intracellular survival in host cells. (A and B) *In vitro* adherence of the TIGR4Δ*cps*Δ*pca* strain to Detroit 562 cells and HBMECs (A) and binding of the D39Δ*cps*Δ*pca* strain by J774 cells (B). The relative adherence and binding efficiencies were correlated to those of the TIGR4Δ*cps* and D39Δ*cps* strains, respectively. (C and D) Invasive properties of the TIGR4Δ*cps*Δ*pca* strain toward Detroit 562 cells and HBMECs (C) and uptake of the D39Δ*cps*Δ*pca* strain by J774 cells (D). The relative invasion and uptake efficiencies were correlated to the number of viable intracellular cells of the TIGR4Δ*cps* and D39Δ*cps* strains, respectively. (E and F) Intracellular survival kinetics of the TIGR4Δ*cps* and TIGR4Δ*cps*Δ*pca* strains in Detroit 562 cells (E) and HBMECs (F). (G) Phagocytic killing of the D39Δ*cps* and D39Δ*cps*Δ*pca* strains in J774 cells. Intracellular survival and phagocytic killing were correlated to viable-bacteria counts at time zero. *, statistically significant differences ($P < 0.05$).

well-characterized homologous β-CA (ECCA) from *E. coli*. Finally, recombinant GST-PCA was able to catalyze the conversion of CO_2 to HCO_3^-. 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. influenza*e 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

Strain[a]	Transformation (CFU/ml) with DNA from:		Growth on or in indicated plate or broth[c]			
	R6	R6Δ*pca*	BA plates	TSA plates	GM17 broth	GM17 broth (0.1% Tween 80)
H23	19,800	14,900	−	−	+	+
H26	67	67	−	−	−	−

[a] Both strains carry the *pca* gene.
[b] No. of colonies growing on BA plates under CO_2-poor conditions.
[c] +, growth; −, no growth.

Our metabolic complementation experiments revealed, in analogy to the role of CAs in other microorganisms (1, 5), that the cellular function of PCA in CO_2-poor conditions is at least linked to FA biosynthesis. This implies that PCA provides HCO_3^- re-

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* Δ*pca* strains in CO_2-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 CO_2 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 *de novo* 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 CO_2, the Δ*pca* strains do not grow, whereas in the presence of CO_2, the Δ*pca* 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 Δ*pca* strains in CO_2-poor conditions with UFAs to restore growth and allow characterization of membrane FAs. However, supplementation of cultures of the pneumococcal wild-type and Δ*pca* 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 H_2O_2, acetyl-phosphate, and CO_2. Production of H_2O_2 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 CO_2 to HCO_3^-, 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* Δ*pca* and Δ*pca*Δ*spxB* 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 Δ*pca* strains in CO_2-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* Δ*pca* 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 HCO_3^- 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 (PAFr) (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 HCO_3^- 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 CO_2-poor conditions, which might be relevant for pneumococcal transmission in environmental ambient air. Detailed examination of the metabolic pathways that depend on PCA-mediated CO_2 fixation and the identification of the genetic basis for the CO_2 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 CO_2- and HCO_3^--poor environments they encounter during transmission, colonization, and disease.

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