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A single amino acid substitution in the MurF UDP-MurNAc-pentapeptide synthetase renders *Streptococcus pneumoniae* dependent on CO₂ and temperature

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Summary

The respiratory tract pathogen *Streptococcus pneumoniae* encounters different levels of environmental CO₂ during transmission, host colonization and disease. About 8% of all pneumococcal isolates are capnophiles that require CO₂-enriched growth conditions. The underlying molecular mechanism for capnophilic behaviour, as well as its biological function is unknown. Here, we found that capnophilic *S. pneumoniae* isolates from clonal complex (CC) 156 (i.e. Spain^{9V}-3 ancestry) and CC344 (i.e. Norway^{NT}-42 ancestry) have a valine at position 179 in the MurF UDP-MurNAc-pentapeptide synthetase. At ≤ 30°C, the growth characteristics of capnophilic and non-capnophilic CC156 strains were equal, but at > 30°C growth and survival of MurF^{V179} strains was dependent

on > 0.1% CO₂-enriched conditions. Expression of MurF^{V179} in *S. pneumoniae* R6 and G54 rendered these, otherwise non-capnophilic strains, capnophilic. Time-lapse microscopy revealed that a capnophilic CC156 strain undergoes rapid autolysis upon exposure to CO₂-poor conditions at 37°C, and staining with fluorescently labelled vancomycin showed a defect in *de novo* cell wall synthesis. In summary, in capnophilic *S. pneumoniae* strains from CC156 and CC344 cell wall synthesis is placed under control of environmental CO₂ levels and temperature. This mechanism might represent a novel strategy of the pneumococcus to rapidly adapt and colonize its host under changing environmental conditions.

Introduction

The Gram-positive bacterium *Streptococcus pneumoniae* is a commensal of the human nasopharynx, with carriage rates of around 10% in adults and over 50% in young infants (Bogaert *et al.*, 2004). In risk groups, mainly young children, the elderly and the immunocompromised, *S. pneumoniae* carriage can lead to invasive pneumococcal disease (IPD). Consequently, this respiratory tract pathogen is a leading cause of community-acquired pneumonia, otitis media, sepsis and meningitis. The existence of over 90 different pneumococcal capsular serotypes implies that capsular polysaccharide (cps)-based vaccines only include and protect against the most prevalent invasive serotypes. As a result, the recent introduction of cps-based vaccines in various national health programmes has successfully reduced the incidence of IPD of the included serotypes, but has also seen the increase in incidence of invasive non-vaccine strains that are often antibiotic resistant (McIntosh and Reinert, 2011). Hence, knowledge on the factors that contribute to the emergence of non-vaccine strains is of vital importance for the design of future therapeutic and prophylactic strategies.

The genetic prerequisite of *S. pneumoniae* strains to persist and spread in the human population is largely unknown. Serotype and antibiotic resistance profiles are important, but expression of other bacterial factors, such as pili, adhesins and toxins, is relevant both for successful

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host nasopharyngeal colonization and for IPD (Kadioglu *et al.*, 2008). There is poor knowledge on other bacterial factors contributing to pneumococcal colonization and pathogenesis. Even less is known about the factors required for bacterial adaptation to the different niches the pathogen can encounter in- and outside the host. Puzzling in this respect is a study by Austrian and Collins in 1966 on naturally occurring capnophilic strains (Austrian and Collins, 1966). It was observed that about 8% of all pneumococcal isolates are capnophiles, suggesting that these strains have lost the ability to survive in the relatively CO₂-poor ambient air conditions it will encounter outside the host, e.g. during transmission. This phenotype was especially pronounced in a few serogroups, i.e. 1, 3, 9, 16 and 28. At the time, the genetic background of capnophilic strains was not assayed. Only recently, we showed that inactivation of pneumococcal carbonic anhydrase (PCA) or dihydrofolate:folylpolyglutamate synthetase (FolC), which catalyse the reversible hydration of CO₂ to bicarbonate and the CO₂-dependent step in the biosynthesis of (polyglutamyl)folate, respectively, can induce a capnophilic growth restriction (Burghout *et al.*, 2010; 2013).

In this study, we examined the genetic background of naturally occurring capnophilic pneumococcal isolates to uncover novel CO₂-dependent cellular functions that could contribute to niche adaptation. We found that the *S. pneumoniae* capnophilic growth restriction could be caused by a single amino acid substitution in the MurF UDP-*N*-acetylmuramoyl-L-alanine-g-D-glutamyl-L-lysyl-D-alanyl-D-alanyl (UDP-MurNAc-pentapeptide) synthetase that is essential for cell wall biogenesis.

Results

S. pneumoniae ST162 strains are capnophilic

To study the genetic background of naturally occurring capnophilic *S. pneumoniae* strains, we performed multilocus sequence typing (MLST) on the capnophilic *S. pneumoniae* H23 and H26 strains that were previously isolated from the nasopharynx of healthy children in Venezuela (Burghout *et al.*, 2010; Quintero *et al.*, 2011). Both strains had sequence type (ST) 162, which means that these strains belong to the pneumococcal clonal complex (CC) 156. The best described member of CC156 is the Spain^{9V}-3 ST156 strain, which is a reference strain in the pneumococcal molecular epidemiology network (PMEN) collection (McGee *et al.*, 2001). To assess if the Spain^{9V}-3 strain is capnophilic as well, growth of this strain was assayed on blood agar (BA) plates that were placed in a 37°C incubator with ambient air, which contains ~ 0.04% CO₂ (i.e. CO₂-poor), or with 5% CO₂-enriched air (i.e. CO₂-rich). As a control we included the non-CC156 *S. pneumoniae* R6 (ST128), G54 (ST63) and TIGR4 (ST205) strains. It

Fig. 1. Growth of capnophilic and non-capnophilic *S. pneumoniae* strains. The *S. pneumoniae* R6, G54, TIGR4, Spain^{9V}-3, H23 and H26 strains were incubated on BA plates that were overnight exposed to CO₂-poor ambient air (A) or 5% CO₂-enriched conditions (B) in a 37°C incubator.

appeared that the Spain^{9V}-3, R6, G54 and TIGR4 strains could be cultured in both CO₂-poor and CO₂-rich conditions, while the H23 and H26 strains could only be cultured in CO₂-rich conditions (Fig. 1). This implies that CC156 contains both capnophilic and non-capnophilic strains.

The pneumococcal capnophilic growth restriction is linked to the gene for the MurF UDP-MurNAc-pentapeptide synthetase

We previously showed that *S. pneumoniae* H23 and H26 genetic transformation with *S. pneumoniae* R6 genomic DNA rendered these strains non-capnophilic (Burghout *et al.*, 2010). This implies that non-capnophilic *S. pneumoniae* strains encode one or more genes that are absent or mutated in capnophilic ST162 strains. To identify the genetic element that can relieve the growth restriction of the capnophilic ST162 strains, we employed transposon

mutagenesis as a genetic mapping tool. In this approach we exploited the fact that co-transformation of two genetic markers, i.e. the transposon antibiotic resistance cassette and the genetic element for non-capnophilic growth, is most efficient when the distance between the two markers on the genome is small. Since the R6 strain does not belong to CC156, we used the non-capnophilic Spain^{9V}-3 strain as donor for genomic DNA. In addition, we used the *S. pneumoniae* BHN100 ST162 strain as a capnophilic reference strain (Sandgren *et al.*, 2005). BHN100 genetic transformation with *in vitro* mariner transposon mutagenized Spain^{9V}-3 genomic DNA yielded six non-capnophilic transposon mutants, i.e. LKIPB08, LKIPB09, LKIPB10, LKIPB11, LKIPB12 and LKIPB13. Sequencing of the transposon insertion sites showed that LKIPB12 acquired DNA from contig 4 of the Spain^{9V}-3 genome, whereas all other strains acquired DNA from contig 2 (Fig. 2A and Table 1). Transformation of BHN100 with genomic DNA of LKIPB08, LKIPB09, LKIPB10, LKIPB11 and LKIPB13 resulted in frequent co-transformation of both markers (Fig. 2A). In contrast, the co-transformation yield with LKIPB12 genomic DNA was below the detection limit. This suggested that the genetic element that can relieve the capnophilic growth restriction of the BHN100 strain is encoded on contig 2 of the Spain^{9V}-3 genome.

To further narrow the Spain^{9V}-3 genomic region that can relieve the capnophilic growth restriction of the BHN100 strain, we used various endonucleases to digest the donor DNA prior to transformation. SphI or BclI digestion of LKIPB09 genomic DNA yielded about 1000 cfu ml⁻¹ non-capnophilic BHN100 transposon mutants (Fig. 2B). In contrast, AgeI and NheI digestion resulted in 60 and < 22 cfu ml⁻¹ non-capnophilic BHN100 transposon mutants respectively. This means that AgeI has reduced the size of the donor DNA needed for homologous recombination of both genetic markers to an absolute minimum, while a NheI restriction site separates the mariner transposon and the genetic element required for non-capnophilic growth. These results suggest that the genetic element that supports non-capnophilic growth is located on the 5.5 kb NheI–AgeI fragment downstream of the mariner transposon in LKIPB09. Based on the Spain^{9V}-3 genome annotation, this DNA fragment contains five genes, namely *pbp2B*, *recR*, *ddl*, *murF* and *mutT*. BHN100 transformation with PCR products of each of these genes demonstrated that uptake of the Spain^{9V}-3 *murF* gene is sufficient to render the BHN100 strain non-capnophilic (Fig. 2C).

A single amino acid substitution in the MurF protein renders pneumococcal strains capnophilic

Amino acid sequence alignment of the BHN100 and Spain^{9V}-3 MurF UDP-MurNAc-pentapeptide synthetase revealed a total of six amino acid changes (Fig. S1). Since

it is likely that at least one of these MurF amino acid modifications is responsible for the observed capnophilic growth defect of the BHN100 strain, we verified the occurrence of these six amino acids in MurF of other *S. pneumoniae* strains. It appeared that Thr-229 and Val-264 are also present in MurF of the non-capnophilic R6, TIGR4 and G54 strains, while Ala-356 is very common and, therefore, unlikely to be linked to capnophilic growth (data not shown). In contrast, Val-179, Phe-215 and Val-315 are unique to MurF in BHN100 and the related SP9-BS68 ST1269 strain. To assay if one of these MurF amino acids is responsible for the capnophilic growth defect of the BHN100 strain, we modified the BHN100 *murF* gene by PCR to introduce Ala-179, Leu-215 or Ala-315 that are present in Spain^{9V}-3 MurF. Next, we used PCR products of the Spain^{9V}-3 *murF*, BHN100 *murF*, BHN100 *murF*^{V179A}, BHN100 *murF*^{F215L} and BHN100 *murF*^{F315A} genes as donor for BHN100 transformation. Transformation with BHN100 *murF*^{V179A} and Spain^{9V}-3 *murF* yielded > 700 cfu ml⁻¹ non-capnophilic BHN100 mutants, whereas the transformation yield was 7- to 10-fold lower with no DNA, BHN100 *murF*, BHN100 *murF*^{F215L} and BHN100 *murF*^{F315A} (Table 2). To confirm that the non-capnophilic BHN100 mutants had taken up the donor DNA, the *murF* gene was sequenced (supplemental Table S2). In case the donor DNA was the BHN100 *murF*^{V179A} or Spain^{9V}-3 *murF* gene, the *murF* gene in the non-capnophilic BHN100 mutants indeed acquired the *murF*^{V179A} mutation. In contrast, the non-capnophilic BHN100 transformants that were obtained with BHN100 *murF*^{F215L} or *murF*^{F315A} donor DNA did not acquire the cognate point mutation. In one case the *murF*^{V179A} mutation had occurred spontaneously. Hence, the presence of a valine residue at position 179 in BHN100 MurF renders this strain capnophilic.

To verify that the presence of the *murF*^{V179} gene is the only prerequisite to render pneumococcal strains capnophilic, we generated *S. pneumoniae* R6 *bga::nisRKΔmurF* and G54 *bga::nisRKΔmurF* strains in which the BHN100 *murF*^{V179}, BHN100 *murF*^{V179A} or Spain^{9V}-3 *murF* gene was provided *in trans* on a plasmid behind a nisin-inducible promoter. Induction of *murF* gene expression by the addition of nisin supported growth of all these strains in CO₂-rich culture conditions (Fig. 3). The strains that expressed the BHN100 *murF*^{V179A} or Spain^{9V}-3 *murF* gene could also be grown in CO₂-poor conditions. In contrast, the presence of the BHN100 *murF*^{V179} gene imposed a capnophilic growth restriction on the otherwise non-capnophilic R6 and G54 strains.

The capnophilic BHN100 strain is temperature-sensitive for growth

The capnophilic growth restriction of the BHN100 wild-type strain is comparable to the temperature-dependent growth

Fig. 2. The capnophilic growth restriction of BHN100 is linked to the *murF* gene. Genetic transformation of BHN100 with: genomic DNA of the Spain^{9V-3}, LKIPB08, LKIPB09, LKIPB10, LKIPB11, LKIPB12 and LKIPB13 strains (A); restriction digested LKIPB09 genomic DNA (B); and the PCR-amplified Spain^{9V-3} *pbp2B*, *recR*, *ddl*, *murF* or *mutT* genes (C). The left part of each panel shows details of the donor DNA. The right part of each panel shows the outcome of the transformation experiment. In (A)–(C), non-capnophilic BHN100 mutants were selected by growth in ambient air at 37°C. In (A) and (B) co-transformation of the transposon was confirmed by selection on spectinomycin (Sp). Triangles and numbers in left part of (A), (B) and (C) indicate the location of the different transposon insertions. In (A), the location of the *mariner* transposon insertion sites are give relative to the 41 contigs of the Spain^{9V-3} genome.

restriction that was previously described for an *Escherichia coli* strain expressing a temperature-sensitive variant of MurF (Lugtenberg and Schijndel-van, 1972). To probe if the capnophilic BHN100 strain is temperature-sensitive as well, we cultured this strain at various temperatures. The BHN100 *murF*^{V179} strain failed to grow and survive in CO₂-poor conditions at 34°C and 37°C, while the isogenic

non-capnophilic BHN100 *murF*^{V179A} strain could readily grow and survive at these temperatures (Fig. 4). Interestingly, BHN100 *murF*^{V179} growth and survival was fully restored at 30°C, indicating that it is temperature-sensitive for growth in a CO₂-poor environment.

Next, we assayed the switching point for the growth of the BHN100 *murF*^{V179}, BHN100 *murF*^{V179A} and the

Table 1. Bacterial strains and plasmids used in this study.

Strain	Relevant feature ^{a,b}	Reference
<i>S. pneumoniae</i>		
AHOY490	ST344; non-typable; capnophilic	Bogaert <i>et al.</i> (2004)
BHN100	ST162; serotype 19F; capnophilic	Sandgren <i>et al.</i> (2005)
G54	ST63; serotype 19F	Dopazo <i>et al.</i> (2001)
H23	ST162; serotype 9V; capnophilic	Burghout <i>et al.</i> (2010); Quintero <i>et al.</i> (2011)
H26	ST162; serotype 9V; capnophilic	Burghout <i>et al.</i> (2010); Quintero <i>et al.</i> (2011)
R6	ST128; unencapsulated	Hoskins <i>et al.</i> (2001)
Spain ^{9V} -3	ST156; serotype 9V	McGee <i>et al.</i> (2001)
TIGR4	ST205; serotype 4	Tettelin <i>et al.</i> (2001)
LKIPB08	BHN100 <i>mariner</i> transposon mutant with Spain ^{9V} -3 DNA; Tn insertion site at bp 124566 in contig 2	This study
LKIPB09	BHN100 <i>mariner</i> transposon mutant with Spain ^{9V} -3 DNA; Tn insertion site at bp 127741 in contig 2	This study
LKIPB10	BHN100 <i>mariner</i> transposon mutant with Spain ^{9V} -3 DNA; Tn insertion site at bp 149796 in contig 2	This study
LKIPB11	BHN100 <i>mariner</i> transposon mutant with Spain ^{9V} -3 DNA; Tn insertion site at bp 118789 in contig 2	This study
LKIPB12	BHN100 <i>mariner</i> transposon mutant with Spain ^{9V} -3 DNA; Tn insertion site at bp 125454 in contig 4	This study
LKIPB13	BHN100 <i>mariner</i> transposon mutant with Spain ^{9V} -3 DNA; Tn insertion site at bp 122038 in contig 2	This study
R6nisRK	Nisin-responsive R6 strain; spr0565::nisRK; Tmp ^r	Kloosterman <i>et al.</i> (2006)
LKIPB14	R6nisRK, Δspr1514 (<i>murF</i>)	This study
G54nisRK	Nisin-responsive G54 strain; SPG_0588::nisRK Tmp ^r	This study
LKIPB15	LKIPB15, ΔSPG_1579 (<i>murF</i>)	This study
LKIPB16	BHN100 <i>murF</i> ^{V179A}	This study
Plasmids		
pCR2.1	Cloning vector; Ap ^r Km ^r	Invitrogen
pBLKI01	pCR2.1 with BHN100 <i>murF</i> ; Ap ^r Km ^r	This study
pBLKI02	pCR2.1 with BHN100 <i>murF</i> ^{V179A} ; Ap ^r Km ^r	This study
pBLKI03	pCR2.1 with Spain ^{9V} -3 <i>murF</i> ; Ap ^r Km ^r	This study
pNG8048E	Expression vector with nisin-inducible promoter; Ca ^r	Kloosterman <i>et al.</i> (2006)
pBLKI04	pNG8048 with BHN100 <i>murF</i> ; Ca ^r	This study
pBLKI05	pNG8048 with BHN100 <i>murF</i> ^{V179A} ; Ca ^r	This study
pBLKI06	pNG8048 with Spain ^{9V} -3 <i>murF</i> ; Ca ^r	This study

a. Tmp^r, trimethoprim resistant; Ap^r, ampicillin resistant; Km^r, kanamycin resistant; Ca^r, chloramphenicol resistant
b. Tn insertion, position of the mariner transposon insertion site in the Spain^{9V}-3 DNA.

Spain^{9V}-3 strains at different levels of environmental CO₂. In a CO₂-depleted environment (i.e. 0% CO₂) at 37°C all three strains failed to grow on BA plates, thus emphasizing the intrinsic importance of adequate levels of environmental CO₂ for pneumococcal growth and survival (Fig. 5A). Ambient air (i.e. 0.04% CO₂) supported growth of the BHN100 *murF*^{V179A} and Spain^{9V}-3 strains, while growth of the BHN100 *murF*^{V179} strain was still impaired. Growth of the capnophilic BHN100 *murF*^{V179} strain was partially

restored with 0.1% CO₂ and fully restored in the presence of 0.2%, 1.0% and 5% CO₂. Broth cultures of these three strains confirmed that the switching point for growth of the BHN100 *murF*^{V179} strain was around 0.2% CO₂ (Fig. 5D–F).

The capnophilic BHN100 murF^{V179} strain is impaired in cell wall biosynthesis

It is likely that the capnophilic growth restriction of the BHN100 *murF*^{V179} strain is caused by a conditional impairment of the MurF UDP-MurNAc-pentapeptide synthetase, which is essential for cell wall biogenesis (Longenecker *et al.*, 2005). To link the capnophilic growth restriction of this strain to a cell wall defect, we incubated BHN100 *murF*^{V179} cultures with various antibiotics. Treatment with the cell wall biosynthesis inhibitors D-cycloserine or vancomycin, resulted in the same growth arrest and rapid loss of bacterial viability as BHN100 *murF*^{V179} exposure to CO₂-poor conditions (Fig. S2). In contrast, treatment of

Table 2. BHN100 transformation with various *murF* variants.

Donor DNA	Non-capnophilic mutants (cfu ml ⁻¹)
Spain ^{9V} -3 <i>murF</i>	745
BHN100 <i>murF</i>	95
BHN100 <i>murF</i> ^{V179A}	700
BHN100 <i>murF</i> ^{F215L}	60
BHN100 <i>murF</i> ^{V315A}	40
No DNA	65

Fig. 3. *MurF*^{V179} renders *S. pneumoniae* R6 and G54 capnophilic. Growth of the R6Δ*murF* (A) and G54Δ*murF* (B) strains expressing BHN100 *murF*^{V179} (squares), BHN100 *murF*^{V179A} (circles), or Spain^{9V}-3 *murF* (triangles) in ambient air (open symbols) or 5% CO₂-enriched (closed symbols) BHI broth medium with 2 ng ml⁻¹ nisin. Growth of the pneumococcal cultures was monitored by recording the optical density at 620 nm (OD₆₂₀).

BHN100 *murF*^{V179} cultures with the protein biosynthesis inhibitor chloramphenicol did not induce a rapid decrease in cell viability.

Finally, we performed time-lapse microscopy on the BHN100 *murF*^{V179} and BHN100 *murF*^{V179A} strains during a temperature shift from 30°C (t = 0) to 37°C (t = 30, 60 and 90 min) in CO₂-poor ambient air conditions (Fig. 6; Movies

S1 and S2). The capnophilic BHN100 *murF*^{V179} strain started to balloon and subsequently lyse directly after the temperature shift, similar to bacteria treated with beta-lactam antibiotics (Yao *et al.*, 2012), while the non-capnophilic BHN100 *murF*^{V179A} strain continued to grow. Next, we shifted low-density (i.e. an optical density at 600 nm of 0.05) BHN100 *murF*^{V179} and BHN100 *murF*^{V179A}

Fig. 4. The capnophilic growth and survival restriction of BHN100 is temperature-dependent. A. Growth of the BHN100 *murF*^{V179} and BHN100 *murF*^{V179A} strains on BA plates incubated in ambient air at 30°C, 34°C or 37°C. B. Survival of the BHN100 *murF*^{V179} (closed symbols) and BHN100 *murF*^{V179A} (open symbols) strains on BA plates incubated in ambient air at room temperature (RT), 30°C, 34°C or 37°C. After 0, 4, 8 or 24 h incubation in ambient air the BA plates were placed in a 5% CO₂ incubator at 37°C to determine remaining viable bacterial counts. Count data are presented as mean values of at least three independent experiments with standard deviation.

Fig. 5. The impact of environmental CO₂ levels on the growth of capnophilic and non-capnophilic *S. pneumoniae* strains. A–C. Growth of the BHN100 *murF*^{V179} (A), BHN100 *murF*^{V179A} (B) and Spain^{9V}-3 (C) strains on BA plates incubated in CO₂-depleted (0%), ambient air (0.04% CO₂), 0.1%, 0.2%, 0.5% or 5% CO₂-enriched conditions at 37°C. D–F. Growth of the BHN100 *murF*^{V179} (D), BHN100 *murF*^{V179A} (E) and Spain^{9V}-3 strains (F) in BHI broth exposed to ambient air (0.04% CO₂), 0.1%, 0.2% or 5% CO₂-enriched (closed symbols) conditions at 37°C. Growth of the pneumococcal broth cultures was monitored by recording the optical density at 620 nm (OD₆₂₀).

cultures from 30°C to 37°C in CO₂-poor ambient air conditions. At 30°C ($t = 0$) incubation with fluorescently labelled vancomycin (VanFL) did not show a different staining pattern for *de novo* cell wall biosynthesis between the strains. However, at 37°C ($t = 15$ min) the VanFL staining pattern of capnophilic BHN100 *murF*^{V179} strain had become asymmetric or had even disappeared, while that of the non-capnophilic BHN100 *murF*^{V179A} strain had remained the same (Fig. 6C and D). Together, these results demonstrate that the *MurF*^{V179} variant results in a lack of *de novo* cell wall synthesis upon high temperatures and low CO₂ conditions.

Capnophilic CC156 and CC344 strains express *MurF* with Val-179

To explore if Val-179 is present in *MurF* of all naturally occurring capnophilic *S. pneumoniae* isolates, an epidemiology study was performed. Included were 487 carriage

and invasive paediatric isolates from 2002, which were collected in the Netherlands before the introduction of the seven-valent pneumococcal conjugate vaccine (PCV-7). We identified 28 (5.7%) capnophilic strains, which associated with seven different clonal complexes (Table 3). Four CCs associated with the international antibiotic-resistant PMEN reference clones Spain^{9V}-3 (CC156), Sweden¹-28 (CC306), Norway^{NT}-42 (CC344) or Netherlands³-31 (CC180) (<http://spneumoniae.mlst.net/pmen/>). Interestingly, 17 out of these 28 strains belong to CC156, most of which have serotype 9V. Sequencing of the *murF* gene showed that all capnophilic CC156 strains and one AHOY490 ST344 strain encoded *MurF*^{V179}. The capnophilic strains from the five other *S. pneumoniae* clonal complexes all encoded *MurF*^{A179}.

To confirm that Val-179 in *MurF* is indeed responsible for the capnophilic growth restriction of the *S. pneumoniae* AHOY490 strain, this strain was transformed with genomic DNA of the non-capnophilic Spain^{9V}-3 or the

Fig. 6. Survival restrictions of the capnophilic BHN100 strain are due to defects in cell wall synthesis. Temperature effect on growth of BHN100 *murF*^{V179} and BHN100 *murF*^{V179A} in ambient air was monitored by time-lapse microscopy. Stills of movies of (A) BHN100 *murF*^{V179} and (B) BHN100 *murF*^{V179A} demonstrate growth behaviour of cells in 30 min steps. Scale bar = 5 μ m. To investigate the effect on peptidoglycan synthesis, cells were pre-grown at 30°C and then shifted to 37°C. Pictures of VanFL-stained cells of (C) BHN100 *murF*^{V179} and (D) BHN100 *murF*^{V179A} were taken just at the time of the temperature shift (T_0) and 15 min after growth on 37°C (T_{15}). Scale bar = 5. Carets point to the absence or to a delocalized VanFL staining pattern.

capnophilic BHN100 strain to provide *murF*^{A179} or *murF*^{V179} respectively. With Spain^{9V}-3 genomic DNA as donor we readily obtained $> 10^6$ cfu ml⁻¹ non-capnophilic AHOY490 transformants, while the yield was < 300 cfu ml⁻¹ when we performed a transformation with no or BHN100 genomic DNA. Sequencing of several non-capnophilic AHOY490 mutants that were obtained after transformation with Spain^{9V}-3 genomic DNA confirmed that the *murF*^{V179A} mutation had occurred (Supplemental Table S2). Interestingly, in two of the non-capnophilic AHOY490 mutants that were obtained after transformation with BHN100 genomic DNA a *murF*^{V179G} mutation had occurred spontaneously.

To examine the occurrence of the different amino acids at the position equivalent to Val-179 in *S. pneumoniae* MurF, 2307 bacterial MurF protein sequences that were

deposited in the NCBI database were aligned. Most MurF homologues have an Ala-179 (70%) or Gly-179 (20%) (Table 4). Val-179 was found in 55 cases (2.4%), and included, besides *S. pneumoniae*, strains of *Helicobacter pylori*, *Pseudomonas* sp., *Borrelia* sp. and *Bartonella* sp. (Supplemental Fig. S3). Cys-179 (3.3%) and Ser-179 (2.6%) were also found on this position, while other amino acids at this site were rare.

Discussion

The respiratory tract pathogen *S. pneumoniae* needs to adapt to the various conditions it encounters during transmission, colonization and disease. Although capnophilic behaviour has been observed for about 8% of all pneumo-

Table 3. Epidemiology of naturally circulating capnophilic *S. pneumoniae* strains.

Strain	Serotype	MLST	Relation to PMEN clone	<i>murF</i> 179	Group
AHOY414	16F	30		Ala	I
AHOY672	16F	30		Ala	
AHOY1395	16F	30		Ala	
AHOY399	16F	5136		Ala	
AHOY16	19F	162	SLV Spain ^{9V-3}	Val	II
AHOY19	9V	162	SLV Spain ^{9V-3}	Val	
AHOY20	9V	162	SLV Spain ^{9V-3}	Val	
AHOY211	19F	162	SLV Spain ^{9V-3}	Val	
AHOY566	9V	162	SLV Spain ^{9V-3}	Val	
AHOY591	9V	162	SLV Spain ^{9V-3}	Val	
AHOY677	19F	162	SLV Spain ^{9V-3}	Val	
AHOY1405	9V	162	SLV Spain ^{9V-3}	Val	
AHOY1708	9V	162	SLV Spain ^{9V-3}	Val	
AHOY2078	19F	162	SLV Spain ^{9V-3}	Val	
AMC2020412	9V	162	SLV Spain ^{9V-3}	Val	
AMC2020821	9V	162	SLV Spain ^{9V-3}	Val	
AMC2021625	9V	162	SLV Spain ^{9V-3}	Val	
AMC2021929	9V	162	SLV Spain ^{9V-3}	Val	
AMC2020010	9V	165	DLV Spain ^{9V-3}	Val	
AHOY391	9V	1142	DLV Spain ^{9V-3}	Val	
AHOY235	19F	3706	DLV Spain ^{9V-3}	Val	
AHOY346a	3	180	Norway ^{NT-42}	Ala	III
AMC2020361	1	228	DLV Sweden ¹⁻²⁸	Ala	IV
AHOY572	38	310		Ala	V
AHOY1474	38	310		Ala	
AHOY490	NT	344	Norway ^{NT-42}	Val	VI
AHOY410	NT	449	DLV Norway ^{NT-42}	Unknown ^a	
AHOY146	NT	3691		Ala	VII

a. The *murF* gene could not be PCR-amplified.
SLV, single locus variant; DLV, double locus variant.

coccal isolates the molecular mechanisms underlying this phenotype are currently unknown. In this study, we revealed that the capnophilic growth behaviour of *S. pneumoniae* strains is a clonal property, which, in CC156 and CC344, is associated with an amino acid substitution in the MurF UDP-MurNAc-pentapeptide synthetase.

MurF belongs to a superfamily of phylogenetically related enzymes that includes the Mur synthetases MurC, MurD, MurE, MurF and Mpl, folypolyglutamate synthetase FolC, cyanophycin synthetase from cyanobacteria, and protein CapB from *Bacillus anthracis* (Eveland *et al.*, 1997;

Ziegler *et al.*, 1998). MurF is a cytoplasmic enzyme, which is involved in the biosynthesis of UDP-MurNAc-pentapeptide. In *S. pneumoniae* this monomeric precursor of peptidoglycan is the result of successive addition of L-Ala, D-Glu, L-Lys and D-Ala-D-Ala to UDP-*N*-acetylmuramic acid by MurC, MurD, MurE and MurF respectively (van Heijenoort, 1996). It was shown that reduced expression of MurF in *Staphylococcus aureus* resulted in the accumulation of the UDP-MurNAc-tripeptide, thinner cell walls and abnormal septa formation during cell division, while absence of MurF proved to be lethal (Sobral *et al.*, 2006). Similarly, the temperature-sensitive *E. coli* MurF2 strain accumulated the UDP-MurNAc-tripeptide at elevated temperatures (Lugtenberg and Schijndel-van, 1972; Eveland *et al.*, 1997). Hence, the growth arrest, autolysis and altered VanFL staining patterns for *de novo* cell wall biosynthesis, we observed at elevated temperature and low environmental CO₂ levels for the capnophilic *S. pneumoniae* BHN100 strain, are in line with an impaired MurF function.

Temperature sensitivity is a phenotype that has been shown for many bacterial mutants, and, most likely, relates to temperature-induced change in the stability of protein

Table 4. Identity of MurF amino acid position 179 in 2307 bacterial strains.

Amino acid ^a	Frequency
Alanine	69.7%
Cysteine	3.3%
Glycine	19.9%
Serine	2.6%
Valine	2.4%
Other	2.2%

a. Equivalent to *S. pneumoniae* MurF sequence.

variants (Lockwood *et al.*, 2011). In contrast, an increased dependence on CO₂-rich conditions has not often been described. Only mutants in the gene coding for carbonic anhydrase consistently depend on CO₂-rich growth conditions (Smith and Ferry, 2000). Carbonic anhydrases catalyse the reversible hydration of CO₂ to bicarbonate, which will facilitate HCO₃⁻/CO₂-dependent metabolic pathways when environmental CO₂ levels are low. Unlike carbonic anhydrases, MurF does not aid the cellular retention of CO₂. Instead, it depends on CO₂ for its enzymatic function. The FolC, MurD, MurE and MurF all have a conserved carbamoylated lysine, i.e. MurF Lys-202, to co-ordinate an active-site metal ion (Bertrand *et al.*, 1999; Dementin *et al.*, 2001; Gordon *et al.*, 2001; Mathieu *et al.*, 2005). It is therefore likely that the observed temperature-dependent capnophilic behaviour of the *S. pneumoniae* BHN100 *murF*^{V179} strain relates to both a temperature-induced change in MurF stability and a change in the carbamoylation state of the Lys-202 residue when environmental CO₂ levels are low. Since both Ala-179 as well as Gly-179 relieve the capnophilic growth restriction of BHN100 MurF and/or AHOY490 with Val-179, it is possible that the larger Val-179 residue has a steric effect on the orientation of Lys-202 to interact with CO₂. This hypothesis is supported by the *S. pneumoniae* MurF crystal structure, which shows that the amino acid at position 179 interacts with the structural α -helix that orients Lys-202 towards the active site (Longenecker *et al.*, 2005).

The physiological need for MurF-induced capnophilic behaviour of *S. pneumoniae* strains remains speculative. The fact that over the last 45 years 5–10% of all pneumococcal strains have consistently been identified as capnophiles (Austrian and Collins, 1966; Flanagan and Paull, 1998), suggests that a capnophilic phenotype has been beneficial for the global spread of specific pneumococcal lineages. This hypothesis is supported by the fact that capnophilic behaviour appears to be clonal. For instance, most capnophilic strains that were identified by Austrian and Collins have the same serogroup, i.e. 1, 3, 9 and 16, as those described in this study (Austrian and Collins, 1966). Therefore we believe that capnophilic behaviour could be one out of the many different successful strategies *S. pneumoniae* have adopted to persist in the human population. MurF^{V179} could act, for instance, as a sensor for the metabolic state of the cells, e.g. by sensing endogenous CO₂ levels, to synchronize cell growth with nutrient availability. Alternatively, the post-translational regulation of cell-wall biosynthesis by adaptation to a CO₂-rich host and CO₂-poor environmental conditions could be beneficial for successful dissemination of the pneumococcus to new hosts. Since cell wall biosynthesis is intimately linked to *cps* biosynthesis (Yother, 2011), a decrease in cell wall biosynthesis would result in a concomitant decrease in the capsule thickness

and, as a result, increased adherence to host cells (Kim and Weiser, 1998; Li-Korotky *et al.*, 2010). After successful colonization, increased MurF activity in the presence of the elevated host CO₂ levels will lead to a thicker capsule and cell wall with positive consequences for bacterial resistance to host immune factors such as muramidases (Davis *et al.*, 2008). This hypothesis would explain the previous observation that a ST162 strain can act as a hypercolonizer (Sandgren *et al.*, 2005). Finally, it is tempting to speculate that MurF^{V179} provides transient resistance to cell wall targeting antibiotics such as penicillin and vancomycin by facilitating the switch to an L-form under antibiotic stress. L-forms are bacteria that, temporarily, lack a cell wall and have been implicated in prolonged infections (Errington, 2013). After the antibiotic exposure, MurF^{V179} cells might readily switch back and restore cell wall synthesis and resume normal growth.

In conclusion, we have revealed a novel functional polymorphism in the MurF UDP-MurNAc-pentapeptide synthetase that is responsible for the growth restriction of most capnophilic pneumococcal strains. This discovery could have important implications for our understanding of pneumococcal cell wall biosynthesis and niche adaptation. The presence of MurF^{V179} in bacterial species that can be capnophilic, e.g. *H. pylori* (Park *et al.*, 2011), or are known to respond to changes in environmental CO₂, e.g. *Borrelia burgdorferi* (Hyde *et al.*, 2007), suggests that this CO₂-dependent molecular mechanism is shared with other species.

Experimental procedures

Bacterial strains and growth conditions

Except for strains used in the epidemiological study, all bacterial strains are listed in Table 1. *S. pneumoniae* was routinely grown in BHI (Difco) with 400 U ml⁻¹ bovine liver catalase (Sigma) or on Colombia agar (Oxoid) plates supplemented with 5% sheep blood in a 5% CO₂ incubator at 37°C. To create CO₂-poor and CO₂-rich growth conditions, broth and plate cultures were exposed to ambient air (0.04% CO₂) or to ambient air enriched with 0.1%, 0.2%, 0.5% or 5% CO₂ respectively. A CO₂-depleted environment was created in a 2 l jar with an open Petris dish filled with 10 ml of a 10 M potassium hydroxide solution. Viable bacterial counts were derived from colony-forming units (cfu) after plating 10-fold serial dilutions of a bacterial culture or cell suspension in 1× PBS on BA plates. Pneumococcal genetic transformation was performed as described previously (Burghout *et al.*, 2007), except that all media were first exposed overnight to 5% CO₂-enriched ambient air. For BHN100 and AHOY490 genetic transformation competence stimulating peptide 1 (CSP-1) (100 ng ml⁻¹) and a 1:1 mixture of CSP-1 (100 ng ml⁻¹) and CSP-2 (100 ng ml⁻¹) were used respectively. The transformation yield has been defined by the number of colonies obtained from 1 ml of competent cells with 100 ng ml⁻¹ donor DNA. Importantly, to compare transformation yields

each set of experiments was performed with the same batch of precompetent cells. *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 (Kloosterman *et al.*, 2006). To select for antibiotic resistance markers, cultures were supplemented with: spectinomycin, 150 µg ml⁻¹; chloroamphenicol, 2.5 µg ml⁻¹ for *S. pneumoniae* and 5 µg ml⁻¹ for *L. lactis*; and nisin, 2 ng ml⁻¹. In the case of antibiotic killing experiments, *S. pneumoniae* was exposed to: chloramphenicol, 34 µg ml⁻¹; d-cycloserine, 50 µg ml⁻¹; or vancomycin, 2 µg ml⁻¹.

General DNA techniques

All plasmids used in this study are listed in Table 1. Oligonucleotides (Biolegio, Nijmegen, the Netherlands) used in this study are listed in Supplementary Table S1. Restriction enzymes (New England Biolabs) were used according to the instruction of the manufacturer. Bacterial genomic DNA was extracted with the Qiagen Genomic tip (Qiagen). For construction of the pPBLK101, pPBLK102 and pPBLK103 plasmids and for the introduction of point mutations in the BHN100 *murF* gene the proofreading Pwo DNA polymerase (Roche) was used. For other PCR-based approaches, AmpliTaq DNA polymerase (Applied Biosystems) was applied. For TA cloning A-overhangs were introduced in PCR products by AmpliTaq DNA polymerase. All PCR reactions were optimized for [Mg²⁺] and annealing temperature, and PCR products were purified with the Qiaquick PCR cleanup kit (Qiagen).

Identification of the *murF* gene in the capnophilic *S. pneumoniae* BHN100 strain

In vitro mutagenesis of Spain^{9V}-3 genomic DNA with pR412T7 as donor for *mariner* transposon was performed as previously described (Burghout *et al.*, 2007). Transposon insertion sites were PCR-amplified by single-primer PCR with PBMrTn8, and PBMrTn1 was used as sequencing primer (Karlyshev *et al.*, 2000). The *pbp2B*, *recR*, *ddl*, *murF* (long), *murF* (short) and *mutT* genes were PCR-amplified with the PBbp2B1/PBbp2B2, PBrecR1/PBrecR2, PBddl1/PBddl2, PBMurF1/PBMurF2, PBMurF3/PBMurF4 and PBMutT1/PBMutT2 primer pairs respectively. To synthesize the BHN100 *murF*^{V179A}, *murF*^{F215L} and *murF*^{V315A} PCR products, we employed a two-step PCR protocol. In the first step, the 5' part of the *murF* gene was PCR-amplified by the PBMurF3/PBMurF9, PBMurF3/PBMurF11 and PBMurF3/PBMurF13 primer pairs, and the 3' part by the PBMurF4/PBMurF8, PBMurF4/PBMurF10 and PBMurF4/PBMurF12 primer pairs respectively. In the second step, the two PCR products were combined to form a single PCR product. For sequencing of the *murF* gene, the gene was PCR-amplified with the PBMurF3/PBMurF4 primer pair, and PBMurF5 was used as a sequencing primer.

Construction of *S. pneumoniae* Δ *murF* strains expressing different *MurF* variants

The *murF* gene of BHN100, LKIPB16 and Spain^{9V}-3 were PCR-amplified with the PBMurF16 and PBMurF17 primer pairs, and cloned into the pCR2.1 cloning vector of the TA

cloning kit (Invitrogen) to obtain pPBLK101, pPBLK102 and pPBLK103 respectively. In the next step, the genes were excised by BsaI/EcoRI digestion and ligated into the NcoI/EcoRI-digested pNG8048 plasmid to obtain pPBLK104, pPBLK105 and pPBLK106 respectively. After introduction of the pPBLK104, pPBLK105 and pPBLK106 plasmids into the *S. pneumoniae* R6nisRK and G54nisRK strains, the genomic copy of the *murF* gene was inactivated by allelic exchange of the target gene with a spectinomycin resistance cassette. Briefly, overlap extension PCR was applied to insert the spectinomycin resistance cassette of the pR412 plasmid between the two 500 bp flanking regions of R6 *murF* gene. The spectinomycin resistance cassette of plasmid pR412T7 and the two 500 bp flanking regions of the R6 *murF* gene were PCR-amplified with the PBpR412_L/PBpR412_R, PBMurF1/PBMurF14 and PBMurF2/PBMurF15 primer pairs. The overlap extension PCR products were transformed into *S. pneumoniae* R6nisRK pPBLK104 strain, and directed mutants were obtained by selective plating in the presence of 2 ng ml⁻¹ nisin. Correct integration of the spectinomycin cassette was confirmed by the induced capnophilic behaviour of the *S. pneumoniae* R6nisRK Δ *murF* pPBLK104 strain. The Δ *murF* mutation was introduced into all other *S. pneumoniae* R6nisRK and G54nisRK strains with pPBLK104, pPBLK105 or pPBLK106 by transformation with *S. pneumoniae* R6nisRK Δ *murF* pPBLK104 genomic DNA as donor.

Selection of naturally occurring capnophilic strains

Streptococcus pneumoniae carriage isolates were sampled previously from healthy individuals in the Rotterdam Area in the Netherlands (Bogaert *et al.*, 2006) and IPD isolates were obtained from the Netherlands Reference Laboratory for Bacterial Meningitis (NRLBM) in Amsterdam, the Netherlands. Only samples from children between 1 and 6 years of age were included in this study. A total of 487 strains, i.e. 382 carriage and 105 IPD strains, were tested for their dependence on CO₂-rich growth conditions in a two step protocol. In the first step, all strains were plated on BA plates and those that had reduced growth in ambient CO₂-poor as compared with CO₂-rich conditions were selected. In the second round of screening, fresh colonies from overnight grown BA plates were resuspended in 1× PBS until 0.5 McFarland, 5 µl was placed on BA plates, and plates were exposed to CO₂-poor and CO₂-rich conditions at 37°C. Strains that presented growth at CO₂-rich conditions but no or only few colonies in CO₂-poor growth conditions were deemed capnophilic.

MLST and sequence alignment

MLST was performed according to a standard procedure (Enright and Spratt, 1998). For identification of allele numbers and sequence types, sequence data were compared with the pneumococcal MLST database (<http://spneumoniae.mlst.net/>) using the program CLCDNA Workbench (CLCbio, Denmark). The Spain^{9V}-3 ST156 genome sequence was derived from NCBI, where a serotype 9V clone from Toulouse is deposited as *S. pneumoniae* SP195. MurF amino acid sequences were derived from NCBI (<http://www.ncbi.nlm.nih.gov>) and aligned with the online CLUSTALW service at the EMBL-EBI (<http://www.ebi.ac.uk>) (Chenna *et al.*, 2003).

Time-lapse microscopy

For time-lapse microscopy cells were pre-cultured in liquid C + Y medium at 30°C and spotted on a low-melting 1.5% C + Y agarose patch. The slide was incubated at 30°C and after 1.5 h the temperature was shifted to 37°C. Phase-contrast pictures were taken every 8 min as described (de Jong *et al.*, 2011).

Fluorescence microscopy

Cells were grown in BHI medium at 30°C to an OD₆₀₀ of 0.05 and then shifted to 37°C. Cells were sampled at different time points. To visualize nascent peptidoglycan, a 1:1 mixture of Bodipy® FL Vancomycin (VanFL) and vancomycin was added at a final concentration of 0.1 µg ml⁻¹ prior to microscopy similarly as described previously (Beilharz *et al.*, 2012). An IX71 Microscope (Olympus), equipped with a 100× phase-contrast objective (1.3 NA) and a CoolSNAP HQ2 camera was used for imaging. To visualize VanFL fluorescence, a GPF filterset (Chroma, excitation 470/40 nm, emission 525/50) was used.

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