Microbes and Infection 19 (2017) 402–412

www.elsevier.com/locate/micinf

Original article

The accessory Sec system (SecY2A2) in *Streptococcus pneumoniae* is involved in export of pneumolysin toxin, adhesion and biofilm formation

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Received 3 February 2017; accepted 17 April 2017
Available online 27 April 2017

Abstract

In *Streptococcus pneumoniae* TIGR4, genes encoding a SecY2A2 accessory Sec system are present within a locus encoding a serine-rich repeat surface protein PsrP. Mutant strains deleted in *secA2* or *psrP* were deficient in biofilm formation, while the Δ*secA2* mutant was reduced in binding to airway epithelial cells. Cell wall protein (CWP) fractions from the Δ*secA2* mutant, but not from the Δ*psrP* mutant, were reduced in haemolytic (pneumolysin) activity. Contact-dependent pneumolysin (Ply) activity of wild type TIGR4 cells was ten-fold greater than that of Δ*secA2* mutant cells suggesting that Ply was not active at the Δ*secA2* cell surface. Ply protein was found to be present in the CWP fraction from the Δ*secA2* mutant, but showed aberrant electrophoretic migration indicative of protein modification. Proteomic analyses led to the discovery that the Δ*secA2* mutant CWP fraction was deficient in two glycosidases as well as other enzymes involved in carbohydrate metabolism. Taken collectively the results suggest that positioning of Ply into the cell wall compartment in active form, together with glycosyl hydrolases and adhesins, requires a functional accessory Sec system.

Keywords: *SecA2*; *Streptococcus pneumoniae*; Pneumolysin; Export; *O*-glycosidase; Biofilms

1. Introduction

Streptococcus pneumoniae, also known as the pneumococcus, colonizes the human respiratory tract. This can be asymptomatic, but in more susceptible individuals e.g. infants, the elderly, immunocompromised, the bacteria can move to other body sites and cause sinusitis, otitis media, pneumonia or meningitis. Pneumococci exhibit many of the properties of oral viridans streptococci, such as α-haemolysis on blood agar caused by hydrogen peroxide production, adherence to glycoproteins [1], biofilm formation [2] and natural competence for DNA-mediated transformation [3]. Components known to be essential for full virulence are the capsular polysaccharides, of which there are more than 90 serotypes [4], choline-binding proteins CbpA, LytA, PcpA, PspA and PspC [5], pore-forming toxin pneumolysin (Ply), and various cell-surface-associated proteins Eno, NanA, PavA, PavB, PsaA and PsrP [6,7].

Ply is a highly conserved 53-kDa pore-forming toxin that is a member of a protein family known as the cholesterol-dependent cytolysins (CDCs). Members of this toxin family are expressed in *Streptococcus*, *Clostridium* and *Listeria*, and

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http://dx.doi.org/10.1016/j.micinf.2017.04.003

include streptolysin O, perfringolysin O and listeriolysin O. The CDC toxins bind to cholesterol in target membranes, and once inserted into the membrane they oligomerize to form pores (350–450 Å in diameter for Ply) [8], resulting in host cell lysis and tissue damage [9]. Ply is produced by virtually all clinical isolates of *S. pneumoniae* and is multifunctional. In addition to cytolytic activity, Ply modulates the immune system by activating the classical complement pathway [10] and neutrophil extracellular trap formation [11]. Ply also affects lysosomal integrity in epithelial cells [12], induces DNA damage and cell cycle arrest [13], and is reported to impact on biofilm formation [14]. However, a confounding issue about the production of Ply is that, unlike other CDC family members, Ply lacks an N-terminal leader peptide to direct secretion through the canonical Sec pathway. Consequently, it has been considered an intracellular protein, released only following cell lysis in vitro or in vivo [15]. More recently, evidence has emerged for autolysis-independent release of Ply [16], and for exported Ply to be localized mainly to the bacterial cell wall [17]. The current notion is that Ply is exported into the cell wall peptidoglycan matrix, within which branch-stem peptides act as a barrier to Ply release [18]. However, the precise mechanism for Ply export across the cell membrane has not yet been established.

Fusion of a canonical Sec signal sequence to Ply did not allow Sec-dependent Ply secretion in *S. pneumoniae*, although secretion occurred when the same construct was expressed in surrogate host *Bacillus subtilis* [19]. More importantly, *B. subtilis* secreted Ply with no leader peptide added, suggesting the existence of a conserved protein export system that is coupled to cell wall localization [19]. In *L. monocytogenes*, listeriolysin O is found extracellularly in complex with a putative chaperone protein (FbpA) and internalin B (InlB) [20]. FbpA protein is one of a number of leader-less proteins in *L. monocytogenes* that are secreted via an alternative pathway known as the accessory Sec system [21]. We hypothesized that the accessory Sec system in *S. pneumoniae* facilitates the export of Ply across the cytoplasmic membrane into the cell wall environment.

In *S. pneumoniae* TIGR4 (serotype 4), *secY2* (transmembrane protein) and *secA2* (ATPase) genes are found within a 37-kb pathogenicity island encoding cell-surface pneumococcal serine-rich repeat protein (PsrP, 4776 aa residues) (Fig. 1). An additional 10 genes encode glycosylation enzymes, and five *asp* genes encode transport complex proteins [22,23]. A similar locus is found in several but not all sequenced pneumococcal genomes. By analogy to genomic loci in *Streptococcus gordonii* and *Streptococcus parasanguinis* encoding serine-rich repeat (SRR) proteins GspB [24], Hsa [25,26] and Fap1 [27], PsrP in *S. pneumoniae* is predicted to become post-translationally glycosylated concomitantly with secretion via the alternate SecY2A2/Asp1-5 translocon [28]. The C-terminus of PsrP glycoprotein is cell wall-anchored while the N-terminal region is projected away from the cell surface and binds keratin [29]. In addition, PsrP plays a role in biofilm formation in vitro [30] and in vivo [31] by mediating direct cell–cell accumulation, or indirectly through binding extracellular DNA [32].

To determine if Ply export was mediated or modulated by the SecY2A2 system in *S. pneumoniae*, the translocon function was inactivated by deletion of the *secA2* gene. The effect of this was to inhibit biofilm formation, association to lung epithelial cells, and export of Ply to the cell wall compartment. Our results suggest that the SecY2A2 translocon is required for efficient localization of Ply to the cell surface.

2. Materials and methods

2.1. Bacterial strains and growth conditions

Bacteria and plasmids used are shown in Table S1. Pneumococci were grown on THY-blood agar (3.6% Todd-Hewitt broth, 5% yeast extract, 12% agar) with 5% defibrinated horse blood, or in THY broth, at 37 °C in 5% CO_2, supplemented as appropriate with 100 μg spectinomycin (Sp) ml^{-1}, 2 μg erythromycin (Em) ml^{-1} or 2 μg chloramphenicol (Cm) ml^{-1}. *Escherichia coli* was cultivated in LB medium, with 100 μg ampicillin (Ap) ml^{-1} or 300 μg Em ml^{-1} as required, and was manipulated by standard protocols. Overnight cultures (10 ml) of all bacteria were grown for approximately 16 h unless stated otherwise. For long term storage (glycerol stocks), overnight cultures were pelleted and resuspended in

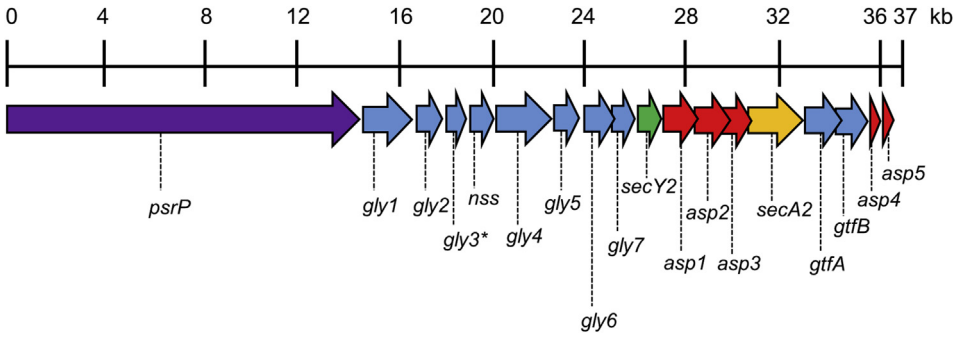

Fig. 1. Schematic representation of the ~36 kb accessory *secY2A2* locus derived from the genome sequence of *S. pneumoniae* TIGR4 (GenBank™ accession number AE005672.3). Genes encode the following: serine-rich repeat protein *psrP* (SP_1772); glycosyltransferases *gly1* (SP_1771), *gly2* (SP_1770), *gly3** (SP_1769), *nss* (SP_1768), *gly4* (SP_1767), *gly5* (SP_1766), *gly6* (SP_1765), *gly7* (SP_1764), *gtfA* (SP_1758, SP_RS08705), *gtfB* (SP_1757, SP_RS08700); accessory secretion proteins *asp1* (SP_1762), *asp2* (SP_1761), *asp3* (SP_1760), *asp4* (SP_1756), *asp5* (SP_1755); *secY2* (SP_1763) and *secA2* (SP_1759, SP_RS08710). *gene contains a frame shift mutation.