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Published in:
P L o S One
DOI:
10.1371/journal.pone.0068484
Publication date:
2013
Document Version
Publisher's PDF, also known as Version of record
Citation for published version (APA):
Complete Genome Sequence of the Cystic Fibrosis Pathogen *Achromobacter xylosoxidans* NH44784-1996 Complies with Important Pathogenic Phenotypes

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Abstract

*Achromobacter xylosoxidans* is an environmental opportunistic pathogen, which infects an increasing number of immunocompromised patients. In this study we combined genomic analysis of a clinical isolated *A. xylosoxidans* strain with phenotypic investigations of its important pathogenic features. We present a complete assembly of the genome of *A. xylosoxidans* NH44784-1996, an isolate from a cystic fibrosis patient obtained in 1996. The genome of *A. xylosoxidans* NH44784-1996 contains approximately 7 million base pairs with 6390 potential protein-coding sequences. We identified several features that render it an opportunistic human pathogen, We found genes involved in anaerobic growth and the *pgaABCD* operon encoding the biofilm adhesin poly-β-1,6-N-acetyl-D-glucosamin. Furthermore, the genome contains a range of antibiotic resistance genes coding efflux pump systems and antibiotic modifying enzymes. *In vitro* studies of *A. xylosoxidans* NH44784-1996 confirmed the genomic evidence for its ability to form biofilms, anaerobic growth via denitrification, and resistance to a broad range of antibiotics. Our investigation enables further studies of the functionality of important identified genes contributing to the pathogenicity of *A. xylosoxidans* and thereby improves our understanding and ability to treat this emerging pathogen.


Editor: Tom Coenye, Ghent University, Belgium

Received January 29, 2013; Accepted May 29, 2013; Published July 22, 2013

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Funding: This work was supported by a grant from the Lundbeck Foundation to TB and by a grant from the Danish Strategic Research Council to MG. CFIM (Core Facility for Integrated Microscopy, The Faculty of Health, University of Copenhagen) is acknowledged for access to SEM. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing interests: The authors have declared that no competing interests exist.

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Introduction

*A. xylosoxidans* formerly known as *Alcaligenes xylosoxidans* is an environmental non-lactose fermenting aerobic motile Gram-negative rod characterized in 1971 [1,2]. It is often found in connection with nosocomial infections targeting immunocompromised patients suffering from cancer, advanced HIV, diabetes mellitus or chronic renal failure [3]. In 1985, the first description of *A. xylosoxidans* in relation to the pulmonary infection of cystic fibrosis (CF) patients was published [4]. CF is the most common lethal autosomal, recessively inherited disease in Caucasians characterized by development of chronic pulmonary infections. Identification of the CF microbiology by 16S rRNA gene sequences has shown diversity in the identification of *A. xylosoxidans* [5] however, two recent investigations have developed multilocus sequence typing (MLST) schemes, which increase the accuracy of identification and characterization of strains and species from the genus *Achromobacter* [6,7]. It is increasingly detected in CF clinics worldwide, with a general incidence of approximately 6-10% [2,8–11]. A retrospective case-control study by Hansen et al. [10] at the Copenhagen CF Centre showed a general increase in the number of CF patients chronically infected with *A. xylosoxidans* from 1 patient in 1993 to 22 in 2005, which
include approximately 8% of all CF patients connected to the
centre. The study indicated a greater decline in lung function
for patients chronically infected with A. xylosoxidans compared
to non-infected patients [10], whereas other studies have not
documented any significant decline in clinical status [2,9].
Generally, A. xylosoxidans is highly resistant to a broad range
of antibiotics including resistance to narrow-spectrum
penicillins, aminoglycosides and several cephalosporins
[12–16], making it difficult to treat. Like most of the other CF
pathogens, A. xylosoxidans can apparently be spread between
CF patients by cross-infection [8,9,17]. All together, these
features characterize A. xylosoxidans as one of the most
important emerging CF pathogens. It is therefore of great
importance to investigate the pathogenicity of A. xylosoxidans
not only to patients with CF but to all immunocompromised
patients suffering from recurring as well as chronic bacterial
infections.

The literature about A. xylosoxidans mostly consists of case
reports, whereas studies documenting important pathogenic
properties and resistance mechanisms are scarce. How A.
xylosoxidans survives and successfully colonize and
chronically infect the CF lung remains to be investigated.
Several factors like biofilm formation, anaerobic growth,
antibiotic resistance and production of exoenzymes and toxins
have been implicated as crucial for some of the more well-
studied CF pathogens such as P. aeruginosa and members of the
Burkholderia cepacia complex to infect and persist in the
lungs of CF patients.

Especially, biofilm formation seems to play an important role
in the persistence of bacteria in chronic CF infections. Biofilm
formation is a growth phenotype many bacteria use for survival
and proliferation in hostile environments, which also leads to
increased tolerance towards antibiotics and host defences
[18–20]. In biofilms, the bacteria aggregate in microcolonies
encased in a matrix consisting of polysaccharides, DNA and
proteins [21]. Biofilm formation in the lungs of chronically
infected CF patients makes treatment strategies very difficult if
not impossible. In the mucus of the CF lung, anaerobic
conditions exist [22,23], which are mainly due to oxygen
depletion by polymorphonuclear leukocytes (PMNs) [62] and
favor survival of bacteria capable of anaerobic respiration.
Some environmental Achromobacter strains have been
documented to reduce nitrate and nitrite to nitrogen gas under
anaerobic growth conditions, and a study of A. xylosoxidans
isolated primarily from ear discharges documented nitrate
reduction [24].

In this study, we sequenced and assembled the complete
genome of an A. xylosoxidans strain isolated from a CF patient
at the Copenhagen CF Centre. The ability of the strain to grow
in O2-depleted environments and to form biofilms was
established by in vitro investigations under different growth
conditions along with studies of the susceptibility of A.
xylosoxidans to a range of antibiotics.

Materials and Methods

Bacterial strains

The A. xylosoxidans strain (NH44784-1996) used for sequencing
was retrieved from sputum from a CF patient (first
time for this patient) of the Copenhagen CF Centre during
routine microbiological assessment in 1996. The strain was
frozen at -80°C in glycerol for preservation. The species
specificity was ensured by both 16S rRNA gene sequence
analysis (Applied Biosystems, California, USA) MALDI-TOF
microseq (Bruker Daltonics, Billerica, USA) and multilocus
sequence analysis (MLSA). Eleven A. xylosoxidans isolates
were retrieved from the same CF patient over a period of 15
years from 1996 to 2011. The following strains were used as
reference in the different in vitro studies; three A. xylosoxidans
reference strains DSM2402, DSM6388 and DSM11852
isolated from an ear discharge, as a contaminant from a
Bacillus culture, and the soil, respectively (DSMZ,
Braunschweig, Germany). P. aeruginosa PAO1 wild-type
obtained from the Pseudomonas Genetic Stock Center
(www.pseudomonas.med.ecu.edu, PAO0001) and Escherichia
coli K-12 [25] were used as comparison in some of the in vitro
investigations.

Sequencing and assembly

A shotgun-sequencing library for titanium chemistry was built
according to the manufacturer’s guidelines (ROCHE) with
some modifications. First, 20 µg DNA was nebulized and
separated on a 0.8% agarose gel. Fragments ranging from 6–
800 bases were excised and used for the adaptor ligation step.
Test emulsion PCR’s were performed to obtain the best copies/
bead ratio. DNA containing beads were then sequenced using
the GS FLX Titanium Sequencing Kit XLR70 on a two region
Titanium Pico Titer Plate (PTP). Two regions were run using
the first Titanium emPCR kit (not including a GC structural
reliever called emPCR additive supplied with the next
generation of emPCR Kits). Additionally, one region was run
using the emPCR additive. To aid scaffold building, a 6 kb
paired-end library was built according to the 3 kb protocol
provided by the manufacturer. In brief, the following
modifications were used: Initially, DNA fragmentation to larger
fragments was performed using nebulization on 15 µg DNA
with the settings of 0.4 bar for 20s. For the circularization
adaptor ligation (step 3.3), only 40 µl of elution buffer EB was
used, which was suitable for the subsequent library span size
selection (step 3.4) that was made from a 1% agarose gel with
subsequent purification using the Qiax II gel extraction kit
(Qiagen). DNA circularization (step 3.6) was performed using
150 ng of filled-in DNA. Finally, for library amplification (step
3.10) 25 cycles were used for the amplification reaction as
compared to only 15 cycles recommended in the
manufacturers protocol. The paired end library was then
sequenced on one region as above, again using the emPCR
additive. The single-end sequencing yielded 549M bp without
emPCR additive, and 257M bp with emPCR additive in total
806M bp. The paired end sequencing yielded 172M bp and an
average insert size of 5,533 bp. The total sequence coverage
was x140.
Assembly was performed with Newbler 2.5p1 (ROCHE) yielding a single 6,957,321 bp scaffold containing 153 gaps. Gap closure was performed using PCR and Sanger sequencing. Due to the high GC content of the DNA, modifications to standard PCR protocols were implemented. This included the addition of PCR additives DMSO (5%), 1.4 M betaine and 5% glycerol (final reaction concentrations) to relieve rigid DNA structures. Originally designed primers had a minimum length of 22 bp and a $T_m$ of 70-73°C, located at a minimum distance of 30 bp upstream or downstream from gaps. PCR was done using Phusion polymerase (Finnzymes), with following program: 98°C for 1 min, then 30 cycles of 98°C for 10s, 65°C for 20s, 72°C for 20s followed by a final extension at 72°C for 10 min. In case of failed PCRs, new primers with $T_m$'s between 81 and 83°C were designed, the annealing and elongation times increased to 45 s, and the number of cycles increased to 35. Sequencing of the obtained PCR products was performed by Macrogen, Korea. Using this approach, 138 gaps were closed. The remaining 15 gaps were located in regions with a GC content above 85% or with highly repetitive sequences including multiple embedded repeats obstructing sequencing.

The sequence was deposited in EMBL bank with the accession number HE798385.

Gene annotation

The software Rapid Annotations using Subsystems Technology (RAST) and SEED [26] were used for annotating the genome of A. xylosoxidans NH44784-1996. The SEED viewer is a framework to support comparative analysis and annotation of genomes. It provides an overview of the basic information such as taxonomy, size, number of contigs, coding sequences, RNAs and non-hypothetical and hypothetical gene annotations. Furthermore, it provides a categorization of the genes in subsystems, which are manually curated and based on functional roles [26,27]. For a more detailed description of the basic technology see 26.

Comparative analysis

I: Comparison of open reading frames (ORFs) by the sequence algorithm BLAST of A. xylosoxidans NH44784-1996 and the 11 most related bacterial strains. The predicted coding regions were used as BLASTP queries into the NR database with an E-value cutoff of 10^-5. The top hits were then sorted by the species they came from and tallied. In cases where the top hit was from another strain of A. xylosoxidans, the species name of the second-best hit was used.

II: Subsystems Tally. The genomes of several relatives of A. xylosoxidans were also annotated with RAST. The subsystems under the 'Resistance to antibiotics and toxic compounds' category were then classified according to the type of resistance they conferred and tallied. The classification is listed in the 'RAST Resistance to Antibiotics and Toxic compounds Subsystems Classification' table.

Phylogenetic analysis

Multilocus sequence analysis (MLSA) was performed as described previously [7]. Sequence chromatograms were edited using ChromasPro (Technelysium Pty. Ltd.) Nucleotide sequence alignments and cluster analyses were made with MEGA5 [28]. Sequences were concatenated using the CLC Main Workbench 6 (CLC Bio). Cluster analysis of concatenated sequences was performed with a neighbour-joining algorithm with 1,000 bootstrap replications. All positions containing gaps and missing data were eliminated. Bordetella petrii DSM 12804 was used as the outgroup.

Phenotypic properties

Growth medium. ABT minimal medium (B medium) [29] plus 2.5 mg thiamine l-1 and 10% A10 [29]) supplemented with 0.5% (wt/vol) glucose and 0.5% (wt/vol) Casamino acids were used for growing the bacteria strains. All strains were incubated with shaking (180 rpm) at 37°C except the three reference strains, which were incubated at 30°C according to recommendations by the provider.

Production of virulence factors

The bacterial strains were grown to an optical density (OD at 600 nm) of 2.0 for up to five days in six-well multi-dishes. Cells were harvested by centrifugation (10,000 rpm for 5 min.) and the supernatant was sterile filtered (pore size 0.22 µm) and thereafter used for detection of virulence factors.

(I): Chitinase assay. Sterile filtered supernatants were mixed with Na-citrate buffer (0.1 M, pH 4.8) in a 2:1 ratio and carboxymethyl-chitin-Remazol brilliant violet (Loewe lochemica GmbH) was added to a final concentration of 0.5 mg/ml. The mixture was incubated on a shaking table (200 rpm) overnight at 37°C, where after the catalyzing reaction was stopped by adding 1M HCL and immediate cooling in ice for 10 min. The mixture was centrifuged at 10,000 rpm for 10 min and the absorbance on a spectrophotometer (Shimadzu, UV-1800) was measured at 550 nm. The values were subtracted from a blank containing NaCl (0.9%) incubated without supernatant.

(II): Elastase assay. Supernatants were mixed with phosphate buffer (0.1 M, pH 6.3) in a 2:1 ratio and Elastin congo Red (Sigma) was added to a final concentration of 2 mg/ml. The mixture were incubated at 37°C with shaken (200 rpm) for 1 week and subsequently centrifuged at 10,000 rpm for 10 min. The absorbance was measured at 495 nm and the values were subtracted from a blank containing NaCl (0.9%) incubated without supernatant.

(III): Protease assay. Supernatants were added to wells made with the back end of a 200 µl pipethead in ABT agar plate (2% agar) containing 5% sterilized skimmed milk. The plates were incubated overnight at 37°C and clearing zones around the wells were measured with a ruler.

(IV): Rhamnolipid. Supernatants were mixed with venous blood from a healthy individual in 25:1 ratio. Hemolysis was visually evaluated as previously described [30] after 20 min and again after 4 hours.
Motility assays

(I): Swarming. 5 µl of 10 x diluted over night culture of each bacterial strain was added on top of an ABT agar plate containing 0.6%, 0.9%, 1.2% or 1.5% bacto agar with 0.2% glucose and casamino acid. The plates were incubated at room temperature, 30°C and 37°C for 24 h.

(II): Swimming. 5 µl of 10 x diluted over night culture of each bacterial strain was added on top of ABT agar plate containing 0.3% agar and 0.2% glucose and casamino acid. The plates were incubated at room temperature, 30°C and 37°C for 24 h.

(III): Twitching. Cells were stab inoculated with a toothpick through a 3 mm ABT agar plate (1.5% bacto agar) containing 0.2% glucose and casamino acid. The plates were incubated at room temperature, 30°C and 37°C for 24 h and 48 h.

Biofilm formation in polystyrene plates

Static biofilm formation was investigated in 96 well polystyrene microtiter plates (Sterilin® Ltd). Overnight cultures were diluted to an OD₆₀₀ of 0.02 in fresh ABT minimal medium supplemented with 0.5% (wt/vol) glucose and 0.5% (wt/vol). Casamino acids were inoculated in 96 well polystyrene microtiter plates and incubated at 37°C (NH44784-1996, PAO1 and E. coli K-12) and 30°C (DSM2402, DSM6388 and DSM11852) for up to 96 hours. Media was removed and wells washed with 0.9% NaCl to remove non-adherent cells. Adherent cells were stained for 30 min. with 0.1% crystal violet (CV) (Sigma) solution and washed twice with 0.9% NaCl to remove non-bound CV. 96% EtOH were added to dissolve bound CV and formation of biofilm were measured using a microplate reader (VICTOR X, PerkinElmer) by determination of OD₆₀₀.

Biofilm formation in flow cells

Biofilms were grown at 37°C in continuous-culture once-through flow chambers perfused with sterile ABtrace minimal medium containing 0.3 mM glucose. The flow chamber system was assembled and prepared as previously described [31]. The development of biofilm was examined after 3 days by confocal laser scanning microscopy (CLSM) (Leica TCS SP5, Leica Microsystems, Germany) equipped with an Argon laser. Images were obtained with a x 40 dry objective and x 100 oil objective. The bacterial viability in the biofilms was assessed by using SYTO 9 [32,33] (Molecular Probes Inc., Eugene, Oreg.) and image scanning was carried out at 488 nm. SYTO 9 was diluted 1000 times in sterile 0.9% NaCl and injected 15 min. before examination by CLSM. To further examine the biofilms in 3D and for generating pictures the IMARIS software package (Bitplane AG, Zurich, Switzerland) was used.

SEM visualization of biofilm formation in a suspension

To visualize the non-surface biofilm aggregating properties of the strains [21] they were cultured under static conditions in six-well multidishes (TPP, Techno Plastic Products AG) in 5 ml LB media for 48 hours at 37°C. The biofilm material was isolated from the supernatant by careful removal of the supernatant by a syringe. The aggregates were harvested and fixed in 2% glutaraldehyde, post-fixed in 1% OsO₄, critical point dried using CO₂ and sputter coated with gold according to standard procedures. Specimens for SEM were investigated with a Philips XL Feg30 SEM microscope operated at 2-5 kV accelerating tension as previously described by 34.

Measurement of nitrous oxide

A. xylosoxidans NH44784-1996 were inoculated from an LB plate in LB media supplemented with either 1 mM or 10 mM KNO₃ or NaNO₃ and grown for 24 and 48 hours in 37°C on a shaking table (180 rpm). O₂ was removed from the media by bubbling with N₂ until anaerobic conditions were established as confirmed with a Multi-parameter Meter HQ40d (HACH Company, Loveland, Co, US). After 24 and 48 hours the concentration of nitrous oxide (N₂O) was measured with an amperometric microelectrode (Unisense A/S) connected to a pA-meter (PA2000, Unisense A/S, Aarhus, Denmark). The microsensor was linearly calibrated at experimental temperature by measurements in N₂O-free medium and medium with known addition of aliquots of N₂O saturated medium. A measurement of N₂O in LB media with no addition of bacteria was subtracted from each of the samples.

Measurements of nitrate and nitrite

The concentration of nitrate and nitrite was measured in the samples grown under anaerobic conditions (see above). The samples were sterile filtered (0.2 µm, Millipore) and the nitrate/ nitrite colorimetric assay kit (Cayman Chemical, Michigan, USA) was used according to the manufacturer.

Pulsed Field Gel Electrophoresis (PFGE)

Genomic profiling using PFGE was performed on the 11 clinical A. xylosoxidans isolates, including the strain used for sequencing. The purified DNA from each strain was digested by use of Spe1 restriction enzyme and the fragments were separated by PFGE following the methods described previously [35,36]. The gels were visually inspected on the Gel Doc™ XR (Bio-Rad) and PFGE patterns were compared by criteria previously published [35–37]. By a difference in 3 or more bands the studied isolates were considered to be unrelated.

Antibiotic susceptibility testing

The minimal inhibitory concentration (MIC) of the clinical isolates was determined for a range of antibiotics using the E-test (Biodisk, Solna, Sweden) according to the instructions of the manufacturer.

Detection of β-lactamase

The 11 clinical isolates and the three reference strains (DSM2402, DSM6388 and DSM11852) were investigated for β-lactamase production by the EDTA nitrocefin test as previously described [38]. A color change to red was visually inspected after 15 min. PAO1 was used as positive controls.
Ethics statement

The Danish Ethics Committee approved the collection of bacteria and informed written consent was obtained from all patients.

Results

The sequenced strain used in this study was collected and isolated from a CF patient at the Copenhagen CF Centre in 1996, during routine bacteriology examination. According to the precipitin data it was the first collected sample after the patient got chronic condition with *A. xylosoxidans* according to the definition of chronically *P. aeruginosa* infections made by Høiby et al. [39].

The complete genome sequence was determined by pyrosequencing on the GS FLX Titanium platform and assembled using the Newbler assembler software. The following annotation analysis of genes is based on the RAST server, an annotation program used for archaeal and bacterial genomes [26]. By using this service it is possible to rapidly gain assessments of gene functions and initial metabolic reconstruction.

Comparative genomic analysis

*A. xylosoxidans* is a member of the β-proteobacteria, and classified as belonging to the order of *Burkholderiales*, which among other families also includes that of the *Burkholderia*. 
The family name of *A. xylosoxidans* is *Alcaligenaceae*, which is also true for the *Bordetella* and *Alcaligenes*. The genus is *Achromobacter*; species *Achromobacter xylosoxidans*; subspecies *xylosoxidans* [41]. Besides *A. xylosoxidans* the genus of *Achromobacter* currently counts the following six species, *Achromobacter denitrificans*, *Achromobacter insolitus*, *Achromobacter marplatensis*, *Achromobacter piechaudii*, *Achromobacter ruhlandii* and *Achromobacter spanius* [http://www.bacterio.cict.fr/].

A comparative analysis of the ORFs for *A. xylosoxidans* and the 11 closest related bacterial genomes by the sequence-alignment algorithm BLASTP revealed that only one organism was the best hit for more than 5% of the ORFs. The environmental Gram-negative *Achromobacter piechaudii* was the source of proteins considered best hits for ~60% of the ORFs (Figure 2).

*A. piechaudii* is very rarely isolated from human infections and only two reports have indicated that *A. piechudii* can act as an opportunistic pathogen [42,43]. *A. xylosoxidans NH-44784-1996* were incorporated in the MSLA dendrogram by Ridderberg et al. [7]. The analysis is generated on the basis of 77 reference and clinical *Achromobacter* strains (see 7 for description of the different strains), which were divided in 5 clusters and the sequenced strain were positioned in group 1 with the type strain of *A. xylosoxidans* (Figure 3).

**Annotation of *A. xylosoxidans* genes.** The outcome of the distribution of genes in different subsystems generated by the RAST software is showed for *A. xylosoxidans NH44784-1996* in Figure 4. PAO1 and *E. coli* K-12 are included for comparison. An important note is that only 47% (*A. xylosoxidans*), 49% (PAO1) and 68% (*E. coli* K-12) of the total amount of genes are included in subsystems. This means that the number of genes presented in Figure 4 will change when all genes present in the genomes are incorporated in the subsystems. Furthermore, the number of genes present in the genome is not identical to the total number of genes incorporated in the different categories of the subsystem. A gene can be part of more than one subsystem and therefore be counted more than one time.

The primary focus for this survey was genes involved in either drug resistance or pathogenicity, such as virulence factors and biofilm formation related genes. The presence of genes found interesting in the *A. xylosoxidans* NH44784-1996 genome was compared to *A. xylosoxidans* A8 (isolated from soil), *P. aeruginosa* PAO1, *B. cenocepacia* J2315 (isolated from a CF patient) and *E. coli* K-12.

**Pathogenic factors.** *A. xylosoxidans* NH44784-1996 harbors several genes involved in functional traits contributing to its ability to colonize and infect humans. We have divided the genes into the following three categories: protein secretion, adhesion, and denitrification (Table 2). All three categories of genes are regarded as being important and contributing to the survival of *A. xylosoxidans* in hostile environments. Regarding important pathogenic factors like exoenzymes and exotoxins contributing to invasiveness, a gene encoding colicin V production is present. Also the gene encoding the exoenzyme regulatory protein AepA precursor is present according to the RAST software. This gene is documented to affect levels of protease and cellulase production [44] and is also present in PAO1.

### Table 1. General features of the *A. xylosoxidans* genome.

<table>
<thead>
<tr>
<th>Chromosome</th>
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<tbody>
<tr>
<td>Genome size (bp)</td>
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<tr>
<td>G+C content</td>
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<tr>
<td>Total genome</td>
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<tr>
<td>RNAS</td>
<td>59.92%</td>
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<tr>
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<tr>
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<tr>
<td>Ribosomal RNAS</td>
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<td>23S</td>
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<td>5S</td>
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<td>Non hypothetical</td>
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<tr>
<td>Total nu. of non hypothetical</td>
<td>4810 (75%)</td>
</tr>
<tr>
<td>Total nu. of hypothetical</td>
<td>1580 (25%)</td>
</tr>
</tbody>
</table>

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Protein secretion

Bacteria use different specialized systems to mediate the transport of molecules out of the cell and into a host cell or the environment. These transport systems have been classified into eight different groups from I to VIII [45] and genes documented to be a part of the following four groups, II, III, VI and VII are present in the *A. xylosoxidans* NH44784-1996 genome. The type II secretion systems (T2SSs) are typical composed of 12 to 16 proteins termed Gsp (General secretion pathway) and are widely conserved among γ-proteobacteria [46]. The system is documented to be involved in the release of extracellular toxins and proteases to the environment [46]. According to the RAST annotation software, 12 genes encoding the T2SS are present in *A. xylosoxidans* NH44784-1996. The same genes are present in *A. xylosoxidans* A8 and *B. cenocepacia* J2315 and 11 of them are present in PAO1 and *E. coli* K-12.

The type III and VI secretion systems (T3SSs and T6SSs) mediate the transport by direct contact with the target cells [47]. The T3SS has much in common with the flagellar export system and delivers virulence factors directly into the host cell [48], which is why this mechanism is found in many pathogenic strains. Thirteen genes encoding the T3SS are found to be present in the *A. xylosoxidans* NH44784-1996 genome as well as in PAO1. Components of the T6SSs are similar to phage tail spike proteins [49]. Twelve genes encoding the T6SS are present in *A. xylosoxidans*, which are also present in *A. xylosoxidans* A8, PAO1 and *B. cenocepacia* J2315. *E. coli* K-12 does not contain the T3SSs or the T6SSs. Three genes encoding sigma-fimbriae proteins categorized to be a part of the type VII secretion systems (T7SSs) by the RAST software, are present in all of *A. xylosoxidans* NH44784-1996, *A. xylosoxidans* A8, PAO1 and *B. cenocepacia* J2315. The particular genes are not present in *E. coli* K-12, but according to the RAST software the organism harbors other genes encoding proteins involved in this secretion system.

Adhesion

The entire pgaABCD locus is present in *A. xylosoxidans* NH44784-1996. This locus which is documented to encode the synthesis of a polysaccharide poly-β-1,6-N-acetyl-D-glucosamine (β-1,6-GlcNAc, PGA) in *E. coli* mediating cell-to-cell and cell-to-surface adhesion for biofilm formation [50]. The cytoplasmic membrane proteins (PgaC and PgaD) are necessary for PGA synthesis and the outer membrane proteins (PgaA and PgaB) are needed for PGA export. *Staphylococcus epidermidis* and *Staphylococcus aureus* also produce β-1,6-GlcNAc polysaccharides, which depends upon the icaABCD locus [51,52]. BLAST analysis of the pga gene products has revealed that several Gram-negative plant and mammalian pathogens have complete homologues to the pgaABCD loci among these are the CF pathogens, *Burkholderia multivorans*, *B. cenocepacia*, *B. cepacia*, *Stenotrophomonas maltophilia*, and *P. fluorescens*. The role of each of the pga genes has been investigated by chromosomal deletions in *E. coli* and showed that all the pga genes are required for optimal biofilm formation [50]. None of the pga genes have been identified in *P. aeruginosa*. 

**Figure 3.** Phylogenetic tree. Neighbor-joining dendrogram showing the relationship of NH44784-1996 and 77 *Achromobacter* strains, using *B. petrii* DSM 12804 as an outgroup. The comparison was based on the concatenated sequences of MLSA genes *atpD, icd, recA, rpoB* and *tyrB* (2,098 nt). MLSA clusters I–V are shown. Bootstrap support of clusters is indicated to the left of the node. Scale bar, 0.01 substitutions per site.
Denitrification

Several genes documented to be present in the \textit{P. aeruginosa} denitrification system are present in the \textit{A. xylosoxidans} NH44784-1996 genome. The \textit{P. aeruginosa} denitrification system is organized in 4 operons, \textit{nar}, \textit{nir}, \textit{nor}, and \textit{nos} encoding 4 reductases that are necessary for the reduction of nitrate to dinitrogen gas [53]. Twelve genes involved in denitrification are present in the \textit{A. xylosoxidans} genome and \textit{A. xylosoxidans} A8 according to the RAST software and 7 of those are present in the PAO1, whereas \textit{E. coli} K-12 or \textit{B. cenocepacia} J2315 do not contain any of the genes encoding denitrification according to RAST. However, \textit{E. coli} has been documented to use both nitrate and nitrite as electron accepter under hypoxic conditions. Of the 12 genes present, 7 are involved in nitrous oxide reductase, 3 are involved in the presence or reduction of nitric oxide and 2 are genes involved in nitrite reductase. The copper-containing nitrite reductase has been shown to be present in \textit{A. cycloclastes} [54] and \textit{Alcaligenes xylosoxidans} [55].

Antibiotic resistance. One important factor for the survival of infectious bacteria is their resistance to administered antibiotics. Several different mechanisms such as enzymatic degradation and efflux pump systems contribute to the resistance patterns of pathogenic bacteria. Genes involved in resistance mechanisms present in \textit{A. xylosoxidans} NH44784-1996 according to the annotation by the RAST software are listed in Table 3.

\textbf{β-lactamases and other antimicrobial modifying enzymes}

The resistance of \textit{A. xylosoxidans} to β-lactam antibiotics is primarily documented by \textit{in vitro} MIC determinations to the
different sub-classes. These investigations have generally shown *A. xylosoxidans* to be resistant to narrow-spectrum penicillins and to several cephalosporins, including cefotaxime, whereas the susceptibility to ureidopenicillins and

<table>
<thead>
<tr>
<th>Product</th>
<th>No. of genes</th>
<th>Gene name/Abbrev.</th>
<th>A8</th>
<th>PAO1</th>
<th>J2315</th>
<th>K-12</th>
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<td>X</td>
<td>X</td>
<td>-</td>
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<td>X</td>
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<td><strong>Denitrification</strong></td>
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<td>X</td>
<td>-</td>
<td>-</td>
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<tr>
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<td>X</td>
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<td>-</td>
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<td>-</td>
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<td>NtrR (Cpf/Fnr family)</td>
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<td>X</td>
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<td>-</td>
<td>X</td>
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<tr>
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<td>X</td>
<td>X</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
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<td>NosX</td>
<td>X</td>
<td>X</td>
<td>-</td>
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</tbody>
</table>

An X refers to the presence of the gene in *A. xylosoxidans* A8, *P. aeruginosa* PAO1, *B. cenocepacia* J2315 or *E. coli* K-12.
Two genes is a class C β-lactamases and four genes a 97% alignment with the class D β-lactamases gene identified in P. aeruginosa sequences of OXA-114 from deposited in GenBank shows have no defined class. In addition, three genes belonging to the metallo-β-lactamase family and two penicillin-binding proteins, identified and characterized from P. aeruginosa strains and was therefore concluded to be naturally occurring among A. xylosoxidans. In addition, it has been detected in 10 clinical isolates [57]. Six sequences of OXA-114 from deposited in GenBank shows a 97% alignment with the class D β-lactamases gene identified in NH44784-1996. This type of β-lactamase is shown in P. aeruginosa to confer high-level resistance to amoxicillin, ticarcillin, piperacillin, cefotaxime, ceftazidime and aztreonam [58].

Two genes is a class C β-lactamases and four genes a 97% alignment with the class D β-lactamases gene identified in P. aeruginosa sequences of OXA-114 from deposited in GenBank shows have no defined class. In addition, three genes belonging to the metallo-β-lactamase family and two penicillin-binding proteins, PBP-2 and PBP-1C has been identified. Acquired β-lactamases belonging to VEB, and the carbapenemases IMP, and VIM have been described [60–62] and furthermore, has a study implicated hyperproduction of β-lactamases by A. xylosoxidans [63]. Three genes encoding aminoglycoside-modifying enzymes are present in A. xylosoxidans NH44784-1996. Aminoglycoside 3'-phosphotransferase (APh(3′VI)), which mediates resistance to kanamycin and several other related aminoglycosides [64]. One predicted aminoglycoside phosphotransferase and one aminoglycoside N3-acetyltransferase, which has been shown to modify a number of aminoglycosides antibiotics including tobramycin and gentamycin [65]. In addition, it has been detected in 10 clinical isolates [57]. Six sequences of OXA-114 from deposited in GenBank shows a 97% alignment with the class D β-lactamases gene identified in NH44784-1996. This type of β-lactamase is shown in P. aeruginosa to confer high-level resistance to amoxicillin, ticarcillin, piperacillin, cefotaxime, ceftazidime and aztreonam [58]. Two genes is a class C β-lactamases and four genes have no defined class. In addition, three genes belonging to the metallo-β-lactamase family and two penicillin-binding proteins, PBP-2 and PBP-1C has been identified.
distributed in nodulation-division (RND)-type efflux pump consisting of an A. xylosoxidans jejuni similarity to NH44784-1996 and the genes are identified as produce extracellular protease was studied by adding sterile filtered supernatants to agar plates containing 5% skimmed milk. A clearing zone is considered as proteolytic activity being present. Chitinase were measured by degradation of chitin according to the gene annotation by the RAST server.

Efflux pump

Several efflux pump systems are present in A. xylosoxidans NH44784-1996 according to the RAST software program. The CmeABC efflux pump system first described in Campylobacter jejuni is one of them [67]. This system is a resistance-nodulation-division (RND)-type efflux pump consisting of an inner membrane transporter CmeB, a periplasmic membrane fusion protein CmeA, and an outer membrane channel protein CmeC [68]. A study by Bador et al. [69] characterized an RND-type efflux pump in an A. xylosoxidans clinical isolate. They identified an operon composed of three ORFs designated AxyABM and a transcriptional regulator situated upstream in an inverted orientation designated AxyR. They showed that the AxyABM efflux system is involved in resistance to several cephalosporins and aztreonam. AxyABM share 97 to 99% similarity to NH44784-1996 and the genes are identified as CmeABC in RAST. In addition, share AxyR 99% similarity to NH44784-1996 and this gene is identified as transcriptional regulator, LysR in RAST and is situated like AxyR. Genes involved in the AcrB-ToIC operon found in E. coli are present. The acrA connected to the AcrAB-ToIC is not present according to the gene annotation by the RAST server.

Comparison of resistance mechanisms

A comparison of the amount of different drug resistance systems as annotated by the RAST software between A. xylosoxidans NH44784-1996 and 10 other pathogens is depicted in Figure 5. The drug resistance systems are divided into the following 3 groups: antibiotic resistance, heavy metal resistance and other resistance. In addition to the genes encoding antibiotic resistance described above, A. xylosoxidans NH44784-1996 contains several genes encoding resistance towards different metals divided into the following systems; copper homeostasis, cobalt-zinc-cadmium resistance, zinc resistance, mercuric resistance, arsenic resistance and resistance to chromium compounds.

Phenotypic properties. Much work has been done to describe the chronic lung infection of P. aeruginosa, which is the most common bacterium found in chronically infected CF lungs. Most of these investigations conclude that P. aeruginosa colonizes and persists in the CF lung due to, e.g. its biofilm forming capacity [70], ability for anaerobic growth [22,71] and production of several virulence factors including rhamnolipids [30,72,73], exoproteases, siderophores, exotoxins and several secondary metabolites. To compare the pathogenic properties of A. xylosoxidans to P. aeruginosa we investigated A. xylosoxidans NH44784-1996 for different pathogenic phenotypic properties known from P. aeruginosa. The ability to produce extracellular protease was studied by adding sterile filtered supernatants to agar plates containing 5% skimmed milk. A clearing zone is considered as proteolytic activity being present. Chitinase were measured by degradation of chitin azure and elastase activity was measured by degradation of elastin congo red. The production of rhamnolipid was visualized by adding sterile filtered supernatant to fresh blood and incubate for 20 minutes. Lysis of the red blood cells indicates the presence of rhamnolipid. By comparison to P. aeruginosa it was clear that none of investigated virulence factors were detectable under the conditions used in this study, indicating that A. xylosoxidans does not rely on the same virulence factors as P. aeruginosa (data not shown).

In vitro biofilm formation

It is believed that the capability of bacteria to persist in chronic infections is due to their biofilm forming capacity, which makes them extremely tolerant towards antimicrobial agents and the host defense [77]. To elucidate whether A. xylosoxidans is also able to use this strategy, we investigated our clinical isolate for its biofilm forming capacity. Initially, we investigated its ability to form a biofilm using polystyrene microtiter wells and the crystal violet biofilm assay [78]. The results of this method strongly depend on the capability of the organism to capable of flagellum-driven swimming motility (data not shown), supporting findings in a previous study [76].
in particular the strain isolated from the soil (DSM11852) showed stronger adherence similar to PAO1 (data not shown).

To investigate biofilm formation under different conditions and to study and characterize the architecture of a possible biofilm in more detail, the clinical isolate was grown in flow chambers with a continuous flow of media. Microscopic inspection of flow cells after 3 days of incubation revealed that *A. xylosoxidans* indeed forms biofilms under the experimental conditions (Figure 6A). However, it appears that the *A. xylosoxidans* biofilm is not formed from the surface with the formation of the mushroom structures often seen in *P. aeruginosa* biofilms [77]. The aggregated *A. xylosoxidans* cells were apparently more suspended. To further study its ability to form aggregates without attaching to a surface *A. xylosoxidans* was grown for 48 hours in a static suspension [21]. This lead to formation of large aggregates within this timeframe. To investigate this aggregation, the aggregated cells were retrieved and visualized by scanning electron microscopy (SEM) (Figure 6B). Again it is very clear that *A. xylosoxidans* forms aggregates with an extracellular matrix, i.e., it forms a biofilm [21,79].

**Anaerobic growth**

The environment in the CF lung is very heterogeneous and it has been shown that O$_2$ levels in the CF mucus are very low or even reach anoxia [22]. Furthermore, the negligible activity of aerobic respiration in fresh sputum samples from infected CF patients (62) suggests anaerobic life-style in the endobronchial mucus, where infecting microbes grow for decades in spite of antibiotic treatment (61). To investigate whether the studied strain is capable of growing in O$_2$-depleted environments, the production of nitrous oxide (N$_2$O), a key intermediate in the denitrification pathway, was monitored under anaerobic growth conditions. PAO1 and *E. coli* K-12 were included as a control (data not shown). After 24 and 48 hours of growth, N$_2$O was present in all the cultures with added nitrate or nitrite. Apparently, *A. xylosoxidans* NH44784-1996 is capable of using denitrification as a respiratory process with both nitrate and nitrite as electron acceptor. LB media contains both nitrate and nitrite and the samples with plain LB media (no addition) showed N$_2$O accumulation after 24 and 48 hours. *E. coli* is a non-denitrifying bacteria however, it has been shown to produce nitrous oxide during nitrate respiration [80]. The content of nitrate and nitrite was investigated in the cultures after the growth periods. In the *A. xylosoxidans* cultures with addition of 1 mM nitrate and nitrite there was no measurable nitrate and nitrite left after 24 h. and in the cultures with addition of 10 mM nitrate and nitrite approx. 2 mM were left after 24 h. More nitrate and nitrite were measured in all the *E. coli* cultures whereas, less nitrate and nitrite were measured in the PAO1 cultures and after 48 h. No nitrate and nitrite were left in the PAO1 cultures (data not shown).

**Antibiotic resistance profile**

To investigate the possible effect of residence periods on the susceptibility of *A. xylosoxidans* to antibiotics, clinical isolates were collected at different consecutive years from the same patient as the *A. xylosoxidans* NH-44784-1996 isolate (Table 4). Eleven isolates collected at different years from 1996 to 2011 were tested for resistance by an E-test towards the following antibiotics and combinations; tobramycin, meropenem, ceftazidime, aztreonam, piperacillin, piperacillin +tazobactam, colistin, ciprofloxacin, trimethoprim +sulfamethoxazole, tetracycline, and chloramphenicol. A PFGE analysis was done in order to evaluate, whether the strains appeared to be of the same clonal origin (data not shown). All strains analyzed from the same CF patient were considered to be of the same clonal origin but of varying subtypes. It should be kept in mind that mutations, inversions or rearrangements in the restriction sites or those creating a restriction site for the specific enzyme used (SpeI) give rise to new PFGE patterns/new subtypes in this analysis. There is a maximum of 2 bands difference between the different isolates and according to Tenover et al. [37] will a difference of 2 to 3 bands between isolates be considered as closely related.

The clinical isolate NH44784-1996 is most susceptible to meropenem, trimethoprim+sulfamethoxazole and the combination of piperacillin and the β-lactamase inhibitor tazobactam, whereas it is resistant as the rest of the isolates to tobramycin, tetracycline, chloramphenicol and the β-lactam..
antibiotic aztreonam. It is previously documented that A. xylosoxidans is innately resistant to aztreonam and aminoglycosides, which corresponds with our data. There was a general increase in resistance towards meropenem and ceftriaxone, whereas several emerging pathogens still need to be studied in detail. New technologies for sequencing and bioinformatics have made it possible to quickly gain a great deal of information and a general overview of the contents of a complete genome and this enables more detailed insights into the genetic repertoire and pathogenicity factors of such emerging pathogens. The size of the A. xylosoxidans genome (6.9 Mbp) is larger than most other CF pathogens (PAO1, 6.3 Mbp [81]; Haemophilus influenza, 1.8 Mbp [82]; S. aureus, 2.8 Mbp [83] and Stenotrophomonas maltophilia, 4.8 Mbp [84]) except some strains belonging to the Burkholderia cepacia complex (Bcc) like, B. cenocepacia [85] and B. multivorans which have genomes larger than 7 Mbp.

De Baets et al. (2007) reported that there was no excessive lung damage or increased decline in lung function in CF patients infected with A. xylosoxidans when compared to the clinical status before acquiring A. xylosoxidans infection. However, recent reports suggest that chronic colonization with A. xylosoxidans had a measurable negative impact on the clinical status and prognosis of CF patients [10,86]. The bacterium has also been shown to rapidly obtain resistance against the major classes of antibiotics, especially aminoglycosides that are widely used in attempts to clear the cystic fibrosis lung from bacterial infection. This emphasizes the need to investigate the molecular pathology of A. xylosoxidans both to find means to control the spread of the increasing number of nosocomial infections, and to combat chronic infections of this multiresistant emerging CF pathogen.

The increased prevalence of A. xylosoxidans in the lungs of CF patients is believed to be related to several factors: The prolonged life expectancy, which leads to an extended evolutionary pressure on the existing microbiota of the CF lungs in response to heavy and continued antibiotic treatment is believed to be one of the underlying reasons that pathogens such as A. xylosoxidans are emerging in connection with CF lung infection [11,87,88]. Another factor is the increased accuracy with which Chromobacter xylosoxidans and other emerging Gram-negative pathogens of the CF lungs are identified [2,89].

Detailed studies of infectious bacterial strains have specified important pathogenic properties necessary for the bacteria to survive and propagate, such as biofilm formation and resistance mechanism towards antibiotics. Since the formal definition of biofilm was first presented [90] the interest in this particular form of bacterial growth has increased immensely. It has become evident that biofilms are most likely the prevalent form of bacterial growth in various chronic bacterial infections [93–95] has only become evident that biofilms are most likely the prevalent form of bacterial growth in various chronic bacterial infections [93–95] has only added to the interest. P. aeruginosa and members of Bcc, which are found to chronically infect lungs of CF patients are known to form biofilms. The biofilm mode of growth in CF lungs enables the bacteria to tolerate both antibiotics administered to patients and attack from the host defense system. The fact that bacteria organized in a biofilm are up to 1000 times more
tolerant towards commonly used antibiotics than their planktonic counterparts [19,94] has thus been an intriguing discovery as well as a considerable driving force in the research on the mechanisms and the regulation of biofilm formation. Our in vitro studies by CLSM and SEM show that *A. xylosoxidans* is capable of aggregating into biofilm like structures. *A. xylosoxidans* biofilm structures have also been detected in sputum samples from CF patients [86]. *A. xylosoxidans* contains the pgaABCD operon, which is documented to encode the polysaccharide β-1,6-GlcNAc that is involved in both cell-cell adherence and cell-surface adherence [50]. Our *in vitro* data supports the possibility of a functional pgaABCD locus in relation to cell-cell adherence, whereas our investigation does not find a strong capability for cell-surface adherence. Different motility patterns have been documented to be necessary for biofilm formation. *A. xylosoxidans* exhibits swimming motility by peritrichous flagella [76], whereas it has not been documented to move either by swarming or twitching motility. This corresponds with our investigation where we found 57 different genes on the *A. xylosoxidans* genome, which are involved in flagellar motility.

Increased hospitalizations of patients with multiresistant bacterial infections highlight the need to understand the precise molecular mechanisms behind the acquisition of antibiotic resistance. This will help limit the development of more multiresistant strains. Several *in vitro* susceptibility tests of clinical specimens conclude that *A. xylosoxidans* is multiresistant [9,16]. *A. xylosoxidans* is commonly sensitive to cefazidime, imipenem, meropenem, piperacillin, carbenicillin, chloramphenicol, and trimethoprim/sulfamethoxazole. On the other hand *A. xylosoxidans* is found to be resistant to a number of antibiotics, including 4-quinolones derivatives, many expanded-spectrum beta-lactams, aztreonam and aminoglycosides in general [8,9,12,76]. However, there is some variation in susceptibility data between different studies presumably due to different treatment methods. Some combinations of antibiotics have shown to increase the antimicrobial activity towards *A. xylosoxidans* including chloramphenicol-minocycline, cipropoloxacin-imipenem, and ciprofloxacin-meropenem [16]. The antibiotic susceptibility observed in earlier studies corresponds very well to our *in vitro* susceptibility tests of *A. xylosoxidans* NH44784-1996 showing general resistance to β-lactams except the carbapenem and meropenem. Three genes encoding class C and D β-lactamases are present in the *A. xylosoxidans* genome, and the presence of functional β-lactamase in all isolates was further indicated by nitrocefin investigations. Except for meropenem, and to some extent ciprofloxacin and trimethoprim/sulfamethoxazole there was not a clear difference in susceptibility between the clinical isolates. However, there were a distinct difference between the reference strain isolated from soil (DSM11852) and the rest of the isolates, which points to an increase in antibiotic resistance after the organism becomes infectious.

The increased amount of nutrient-rich mucus in a CF lung generates a perfect growth environment for bacteria. Oxygen is consumed rapidly in the mucus by the activated PMNs for the respiratory burst (62) leading to the formation of O₂ concentration gradients [22]. Such hypoxic or even anoxic microenvironments allow facultative anaerobes such as *P. aeruginosa* to persist. The presence of strict anaerobes within the lungs of CF patients has also been demonstrated [96]. In the present study we showed that the clinical *A. xylosoxidans* strain was able to respire nitrate and nitrite under anaerobic conditions as indicated by its production of N₂O. We also demonstrated the presence of denitrification genes in the *A. xylosoxidans* genome, which are similar to those in *P. aeruginosa* [53]. The capability of *A. xylosoxidans* to grow in anaerobic conditions in the CF sputum can also explain the inflammatory response that was found to characterize the chronic infection that correlates to a rapid decline in lung function [10,86].

In conclusion, we have sequenced and assembled the genome of the emerging pathogen *A. xylosoxidans*, however the sequence still contains 15 gaps, which cannot be assembled at present. We have identified a series of genes important for the organism to survive and proliferate in hostile environments. The different phenotypic traits discussed above are key factors allowing *A. xylosoxidans* to persist as a chronic infection in the lungs of CF patients regardless of the host defense, aggressive antibiotic treatments and the presence of other bacterial species. Increasing knowledge about the molecular details of pathogenic *A. xylosoxidans* strains will lead to better understanding of the increase in numbers of infections and thereby lead to improved treatment strategies.

**Author Contributions**

Conceived and designed the experiments: THJ MAH PØJ LH LR AC MK WR CRH SE MH PK MA KQ MB CM MK OC MG SJS NH TB. Performed the experiments: THJ MAH PØJ LR AC WR MK SE MH PK MA KQ OC MB CM MK OC MG SJS NH TB. Contributed reagents/materials/analysis tools: MAH PØJ KQ MK OC MG SJS NH TB. Wrote the manuscript: THJ MAH PØJ LH LR AC WR MK CRH SE MH PK MA KQ MB CM MK OC MG SJS NH TB.

**References**


Complete Genome Sequence of A. xylosoxidans

Complete Genome Sequence of A. xylosoxidans


