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Research paper

Isolation and whole genome analysis of endospore-forming bacteria from heroin

Jörn Kalinowski[a], Björn Ahrens[b], Arwa Al-Dilaimi[a], Anika Winkler[a], Daniel Wibberg[a], Uwe Schleenbecker[b], Christian Rückert[a], Roman Wölfel[c], Gregor Grass[c,*]

[a] Bielefeld University, Center for Biotechnology, Universitätsstraße 27, 33615 Bielefeld, Germany
[b] Federal Criminal Police Office, Forensic Science Institute, Äppelallee 45, 65203 Wiesbaden, Germany
[c] Bundeswehr Institute of Microbiology, Neuherbergstraße 11, 80937 Munich, Germany

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ABSTRACT

Infections caused by endospore-forming bacteria have been associated with severe illness and death among persons who inject drugs. Analysis of the bacteria residing in heroin has thus been biased towards species that affect human health. Similarly, exploration of the bacterial diversity of seized street market heroin correlated with the skin microflora of recreational heroin users insofar as different *Staphylococus* spp. or typical environmental endospore formers including *Bacillus cereus* and other Bacilli outside the *B. cereus sensu lato* group as well as diverse Clostridia were identified. In this work 82 samples of non-street market ("wholesale") heroin originating from the German Federal Criminal Police Office's heroin analysis program seized during the period between 2009 and 2014 were analyzed for contaminating bacteria. Without contact with the end user and with only little contaminations introduced by final processing, adulteration and cutting this heroin likely harbors original microbiota from the drug's original source or trafficking route. We found this drug to be only sparsely populated with retrievable heterotrophic, aerobic bacteria. In total, 68 isolates were retrieved from 49 out of 82 samples analyzed (60% culture positive). All isolates were endospore-forming, Gram-positive Bacilli. Completely absent were non-endospore-formers or Gram-negatives. The three most predominant species were *Bacillus clausii*, *Bacillus* (*para*)*licheniformis*, *and Terribacillus saccharophilus*. Whole genome sequencing of these 68 isolates was performed using Illumina technology. Sequence data sets were assembled and annotated using an automated bioinformatics pipeline. Average nucleotide identity (ANI) values were calculated for all draft genomes and all close to identical genomes (ANI > 99.5%) were compared to the forensic data of the seized drug, showing positive correlations that strongly warrant further research on this subject.

1. Introduction

Bacterial endospores are remarkably capable of surviving harsh environments because they are intrinsically resistant to stresses such as extreme temperatures, desiccation, ionizing radiation, broad ranges of pH-values, disinfectants and other chemicals, *etc.* [1]. The most prominent endospore formers belong to the Gram-positive Bacilli family. These microorganisms are typically inhabitants of soil where desiccation and other physical stresses are frequently reoccurring phenomena. Because of their environmental resilience, endospore formers have also been detected in illegal drugs such as heroin [2–6]. Probably the first thorough survey of microorganisms from heroin and the drug users' paraphernalia reported that two thirds of the heroin tested samples were culture positive. The most abundantly recovered bacteria from these samples were *Bacillus* species followed by other Gram-positives [2]. Generally, the largest group of microbes recovered from heroin comprises endospore formers, followed by non-endospore forming Gram-positives, and, to a lower degree, fungi. Conversely, Gram-negative organisms were rarely recovered [2–6].

Infections in people who use drugs often have their origins from microbial contamination in the drug itself, drug paraphernalia, from the user's drug preparation practices or from the individual's microflora [7]. Such infections may be caused by *Bacillus* species contaminating heroin and injection materials leading to *Bacillus*-caused diseases ranging in clinical severity from asymptomatic bacteremia to life-threatening endocarditis or endarteritis [8] and, more rarely, to injectional anthrax [9,10]. Strains associated with a large number of injectional anthrax cases have been recently recognized by microbial bioforensics as highly related, originating from a common but yet uncharted geographic source [11]. In parallel, efforts have been made to retrieve

* Corresponding author.
E-mail address: gregorgrass@bundeswehr.org (G. Grass).

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Bacillus anthracis from seized heroin [10,12] but thus far without avail. Notwithstanding, these efforts made us aware of the potential of analyzing bacteria contaminating the drug in concert with traditional forensics for attribution purposes.

Recently, we conducted a study aimed at retrieving assumed *B. anthracis* contamination in 82 samples of seizures from illegal "wholesale" levels. These samples originated from the German Federal Criminal Police Office's German Heroin Analysis Programme seized during the period between 2009 and 2014 [12]. In the present study, we explored the potential bioforensics utility of characterizing bacteria from these heroin samples. Without contact to the street market we anticipated this heroin to harbor microbiota representative to the drug's original source, processing facilities or trafficking route with only limited opportunities of contaminations introduced during further processing, adulteration and cutting activities. Importantly, these samples would deliver distinct microbiota which would be independent of that of any end-user's commensal microflora.

The organisms contaminating the drug samples were analyzed on a genome-wide level and were used for preliminary criminal attribution purposes. These forensic efforts can be expected, with a growing database, to aid in elucidating distribution networks and geographic origins of heroin-processing by overlaying relationships of bacterial genomic data with that of traditional criminal forensics.

2. Material and methods

2.1. Heroin samples

The 82 heroin samples provided by the German Federal Criminal Police Office originating from the German Heroin Analysis Programme were the same as in [12]. All samples were collected in the time period between 2009 and 2014. Their diacetylmorphine concentrations ranged from 24.9% to 69.8% (w/w) determined as described in [13].

2.2. Extraction of bacteria and cultivation

Except for initial direct plating experiments, we followed our previously published method [12]. In short, each heroin sample (1–2.5 g) was mixed 1–5 (w/v) with Heroin Wash Solution (HWS, sterile dH_20 with 0.5% (v/v) Tween 20) and thoroughly vortexed for 5 min in order to separate microorganisms from the organic matrix. The heroin was removed by low speed centrifugation for 2 min at 2000 rpm (555 × *g*). Next, the supernatant was transferred into growth media (double strength LB broth) in a volume of approximately 1–2 (supernatant to growth media). Though expecting typical environmental microorganisms, we conducted the following steps involving live bacteria in a class II biosafety cabinet. The enrichment culture was incubated with aerobe shaking at 37 °C for 24–72 h or until turbid (maximum 1 week). Finally, samples were diluted and plated on different solid growth media (regular solidified Lysis Broth media, LB agar) as well as Columbia blood agar (both Becton, Dickinson and Company). Cultures were grown at 37 °C for 16 h (up to 48 h) until colonies formed. Colonies were re-streaked on the same type of agar and grown. Then cultures were conserved at −80 °C using Microbank cryotubes (Pro-Lab Diagnostics). Throughout, the disinfectant used was 2% Terralin PAA (Schülke & Mayr) with peracetic acid as active ingredient for efficient inactivation of bacteria and their endospores [14]. Terralin PAA was also used for disinfection and to denature heroin samples after experimental use and prior to thermal disposal.

2.3. Inactivation of bacteria and extraction of DNA

In general, bacteria were inactivated (for DNA extraction) after a single passage following isolation on agar in order to minimize culturing-related genetic changes. Where needed, cultures were grown from −80 °C stocks and colony material retrieved from this agar plate directly. For each isolate a few colonies were scraped off the agar plate and transferred into a 1.5 ml reaction tube filled with 500 µl fresh 2% (v/v) Terralin PAA and the cell pellet was carefully resuspended by pipetting up and down. The reaction tube was completely filled up with 2% (v/v) Terralin PAA and incubated for 30 min for inactivation of vegetative cells and endospores. Following centrifugation at 6000 × *g* for 2 min the supernatant was removed and the pellet carefully resuspended in 1 ml phosphate-buffered saline (PBS). After two washing steps with 1 ml PBS the pellet was stored at −20 °C until further use.

For isolation of DNA from inactivated bacterial suspensions, the DNeasy Blood and Tissue Kit (Qiagen) was used as described in the manufacturer's manual for Gram-positive bacteria with minor changes described in [15]. The DNA concentrations of each eluate were quantified using the Qubit dsDNA HS Assay Kit (Thermo Fisher) according to the supplier's protocol. DNA solutions were stored at −20 °C until further use.

2.4. Preliminary species identification of the isolates

The ribosomal small subunit-encoding gene (16S rRNA gene) was amplified by polymerase chain reaction (PCR) as previously described [16]. The individual 16S rRNA gene fragments were subjected to DNA sequencing without prior cloning using the same set of primers. Reads belonging to individual strains were aligned using Bionumerics 6.6. (Applied Math) and the sequence subjected to BLAST search [17].

2.5. Library preparation, genome sequencing and phylogenetic analysis

For each of the 68 isolates a paired-end library (2 × 100 nt) was generated (Nextera XT DNA Library Preparation Kit, Illumina) and sequenced on a Illumina HiSeq 1500 sequencer. The resulting read pairs were assembled *de novo* using the Roche GS *De Novo* Assembler Software (version 2.8). A first phylogenetic analysis of the sequenced bacteria was conducted based on 16S rRNA gene sequences extracted from the assembled genomes and compared to the Ribosomal Database Project (RDP) database [18]. In addition, for in-depth analysis of the genome sequence relationships between the isolates further phylogenetic analysis was carried out based on calculating pairwise average nucleotide identities (ANI) [19,20] using the entire scaffold set of each genome. The ANI data were converted to a phylogenetic tree using the cluster 3.0 and TreeView tools (http://bonsai.hgc.jp/~mdehoon/software/cluster/software.htm).

The genome data have been deposited in GenBank, the accession numbers are given in Supplementary Table 1.

3. Results

3.1. Seized heroin harbors a limited but distinct set of bacteria comprising endospore-forming Firmicutes of the order Bacillales

Analysis of all 82 heroin samples yielded 68 isolates retrieved from 49 culture-positive heroin samples (60% culture positive). Thus, the bacterial load of this heroin was quite low. Typically, culturing from the drug had to be carried out for at least two days before growth was observed. Because of the low contaminant numbers we did not quantify the colony forming units per gram of heroin. Morphological inspection of the growth after spreading on solidified media yielded 13 samples containing more than one species. One sample produced five, one sample four, one samples three and nine samples two different isolates. Species identification by (partial) 16S rRNA gene sequence analysis revealed that all isolates belonged to the Gram-positive, endospore forming order Bacillales comprising the families Bacillaceae and Paenibacillaceae (data not shown).

In order to provide higher taxonomic resolution and to delineate finer genetic relationships between the 68 isolates, a genome-sequencing and -analysis approach was applied. For this, draft whole genome