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SHORT COMMUNICATION

Random transposon mutagenesis of *Verrucomicrobium spinosum* DSM 4136^T

Daryl B. Domman · Blaire T. Steven · Naomi L. Ward

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Abstract The Verrucomicrobia are a bacterial group of growing interest due to their environmental ubiquity as free-living and host-associated microbes. They also exhibit an unusual compartmentalized cell plan, shared with members of neighboring phyla that include the Planctomycete bacteria. However, Verrucomicrobia are currently difficult to study, due to a lack of available genetic tools that would permit robust testing of hypotheses formulated from ecological and genomic data. To our knowledge, there are no published studies describing the transformation of exogenous DNA into any members of the Verrucomicrobia (or the neighboring phylum containing Planctomycetes). Here, we present a procedure for the transformation of DNA into *Verrucomicrobium spinosum* DSM 4136^T via electroporation and the first description of a random transposon mutant library in this organism. We anticipate that this approach could be applied successfully to other Verrucomicrobia, providing opportunities to test the role of predicted gene function in ecological interactions and identify genes associated with the distinctive Planctomycete–Verrucomicrobial cell plan.

Keywords Verrucomicrobia · *Verrucomicrobium spinosum* · Transformation · Transposon · Mutant

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D. B. Domman and B. T. Steven contributed equally to this work.

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D. B. Domman · B. T. Steven · N. L. Ward (✉)
Department of Molecular Biology, University of Wyoming, Dept 3944, 1000 E. University Ave, Laramie, WY 82071, USA
e-mail: nlward@uwyo.edu

N. L. Ward
Department of Botany, University of Wyoming, Laramie, WY 82071, USA

N. L. Ward
Program in Ecology, University of Wyoming, Laramie, WY 82071, USA

Introduction

The bacterial phylum 'Verrucomicrobia' (Schlesner et al. 2006; Yoon et al. 2010b) is of growing interest due to its widespread distribution (Ward-Rainey et al. 1995; Hugenholtz et al. 1998; Janssen 2006) and unusual host associations (Vandekerckhove et al. 2000; Petroni et al. 2000; Derrien et al. 2004). Verrucomicrobia also exhibit a compartmentalized cell plan (Lee et al. 2009) shared uniquely with the neighboring phyla of Planctomycetes and Poribacteria (Wagner and Horn 2006) and recently implicated in eukaryotic-type endocytosis and vesicle trafficking (Santarella-Mellwig et al. 2010; Lonhienne et al. 2010). The phylum name derives from the species *Verrucomicrobium spinosum* (Schlesner 1987) and is associated with higher taxa that include the Class Verrucomicrobiae, Order *Verrucomicrobiales*, and Family *Verrucomicrobiaceae* (Ward-Rainey et al. 1995; Hedlund et al. 1997; emend. Yoon et al. 2008a), as well as the proposed Division 'Verrucomicrobia' (Hedlund et al. 1997). The term "division" is used rather interchangeably with "phylum" to describe higher-level taxa in certain parts of the bacterial tree, but like other terms above the rank of class is not governed by the Bacteriological Code (Lapage et al. 1992).

In the past year alone, Verrucomicrobial sequences have been reported from the intestinal tracts of earthworms (Wüst et al. 2010), cockroaches (Berlanga et al. 2009), and leeches (Schulz and Faisal 2010); Amazonian rainforest soils (Grossman et al. 2010); a humic lake (Arnds et al. 2010); rhizosphere in heavy metal contaminated soil (Navarro-Noya et al. 2010); and cave walls (Pasić et al. 2010). These environmental sequences are complemented by a rapidly expanding group of new Verrucomicrobial isolates (Sangwan et al. 2004; Kasai et al. 2007; Yoon et al. 2007a, b, c; Dunfield et al. 2007; Yoon et al. 2008a, b; Bibi et al. 2010; Yoon et al. 2010a, b). The ecological roles of Verrucomicrobia are still largely unknown. Some Verrucomicrobial species are capable of degrading plant polymers (Janssen et al. 1997; Chin et al. 1999), methane oxidation (Dunfield et al. 2007; Pol et al. 2007; Islam et al. 2008), or nitrogen fixation (Khadem et al. 2010).

Despite their intriguing properties, Verrucomicrobia are difficult to study due to a lack of genetic tools that would permit robust hypothesis testing. This is a problem that extends beyond the Verrucomicrobia to other members of the Planctomycetes–Verrucomicrobia–Chlamydiae (PVC) superphylum (Wagner and Horn 2006). For example, to date, there are no published studies describing the stable introduction of exogenous DNA into any members of the Planctomycetes. Even in the more heavily studied Chlamydiae, development of genetic tools is very recent. Transient transformation of *Chlamydia trachomatis* was achieved via electroporation of elementary bodies with recombinant plasmid DNA (Tam et al. 1994), but stable maintenance of plasmids allowing directed mutagenesis of chlamydial genomes is much more recent (Binet and Maurelli 2009). Here, we report (to our knowledge) the first introduction of exogenous DNA and first generation of a random mutant library, in a member of the Verrucomicrobia. We tested the EZ-Tn5 transposome system (Epicentre Biotechnologies) due to its reported high-efficiency and transposition in various bacterial systems (Goryshin and Reznikoff 1998; Davies et al. 2000; Fernandes et al. 2001).

Materials and methods

Bacterial strains and culture conditions

V. spinosum DSM 4136^T was obtained from the DSMZ and maintained aerobically at 30°C in M13 liquid medium (Schlesner 1987; DMSZ Medium 607) with shaking at 175 rpm. VL70 minimal medium (Schoenborn et al. 2004) for the identification of mutants unable to grow on glucose as a sole carbon source was prepared with the following per liter: 2.09 g 3-[*N*-morpholino]propanesulfonic acid; 20 mM $MgSO_4$; 30 mM $CaCl_2$; 20 mM $(NH_4)_2HPO_4$; 1 ml selenite–tungstate solution (Atlas 1995); 1 ml SL-10 trace element solution (Atlas 1995). After autoclaving, 3 ml of vitamin 10 mix (Janssen et al. 1997) was added along with 2 mM (final concentration) filter-sterilized glucose. Kanamycin sensitivity testing was performed by inoculation onto M13 agar plates supplemented with kanamycin sulfate (1–100 μg ml^{-1}, in 10 mg ml^{-1} increments, each concentration in triplicate).

Transformation

One milliliter of culture grown to an OD_{600} of 0.15 as described above was centrifuged (13,000 rpm; 12, 500×*g*) for 1 min. The resulting cell pellet was washed twice in 1 ml sterile 10% (w/v) sucrose, resuspended in 50 μl 10% w/v sucrose, and mixed with 40 ng (2 μl) of EZ-Tn5 (KAN-2) Tnp Transposome (Epicentre Technologies). The resulting mixture was transferred into a chilled 1-mm electrode gap electroporation cuvette (Life Science Products Inc.), and electroporation performed using an Eppendorf Electroporator 2510 under the following conditions: 1.0 KV, 200 Ω, and 25 μF. Cells were mixed immediately with 500 μl M13 medium, transferred to a 1.5-ml microcentrifuge tube, and incubated at 30°C with shaking (175 rpm) for 2 h to allow recovery and expression of antibiotic resistance genes. Cells were subsequently centrifuged (13,000 rpm; 12,500×*g*) for 1 min, and the resulting pellet was resuspended in 400 μl M13 medium. After plating 100 μl aliquots onto solid M13 medium with or without the addition of 40 μg ml^{-1} kanamycin, plates were incubated for 4 days at 30°C.

Confirmation and mapping of transposon insertions via PCR and inverse PCR

Individual colonies were inoculated into 5 ml M13 broth supplemented with 40 μg ml^{-1} kanamycin and incubated as described above. The bacterial pellet obtained by centrifugation (13,000 rpm; 12,500×*g*) of 1 ml of culture was used for genomic DNA extraction with the DNeasy Blood and Tissue Kit (Qiagen), according to manufacturer's recommended protocols. We confirmed presence of the Tn5 within chromosomal DNA via PCR, by amplifying a 1,109 bp product using primers specific to the Tn5 sequence: EZ-Tn5-F (5′-GGTTGATGAGAGCTTTGTTG TAGGT-3′); EZ-Tn5-R (5′-CTCAAAATCTCTGATGTT ACATTGC-3′). Cycle conditions were as follows: 95°C for 4 min; 30× (95°C for 30 s, 54.5°C for 20 s, 72°C for 2 min); 72°C for 10 min, 10°C hold on a Bio-Rad iCycler. PCR products were examined via standard agarose gel electrophoresis.

Inverse PCR (iPCR) as described by Fernandes et al. (2001) was adapted to map transposon mutants in

V. spinosum. Briefly, 20 µl of genomic DNA isolated from putative transformants as described above was digested with HpyCH4IV restriction endonuclease (New England Biolabs) for 2 h at 37°C followed by heat inactivation at 65°C for 10 min. The remainder of the iPCR was performed as described by Fernandes et al. (2001), using outward primers from the transposon (KAN-2FP-1: 5′-ACCTACAACAAAGCTCTCATCAACC-3′; HpyTn5-Outward: 5′AATTTAATCGCGGCCTCGAGC-3′).

DNA sequence analysis

Inverse PCR products were gel purified using the Sigma GenElute Gel Extraction Kit. Sequencing reactions were performed at Sequetech (www.sequetech.com) using the KAN-2-FP-1 primer and BigDye Terminator on an ABI 3730xl DNA Analyzer platform. One iPCR amplification product (from transformant VSDD21) was gel purified, cloned into pCR2.1-TOPO (Invitrogen), and chemically transformed into TOP10 *E. coli* (Invitrogen) cells, according to the manufacturer's recommended protocols. The recombinant plasmid was then sequenced using the M13-Forward primer (5′-GTAAAACGACGGCCAG-3′). Sequence data were edited using MacVector (MacVector Inc) and Sequencher (Gene Codes Corp). Sequences were located within the *V. spinosum* DSM 4136^T genome using tBLASTx against the NCBI database, and gene annotations were obtained from the Integrated Microbial Genome database (Joint Genome Institute). The genome map for Online Resource 1 was generated using CGViewer (Stothard and Wishart 2005).

Results and discussion

Determination of kanamycin sensitivity profile

Wild-type growth assessed after 5 days incubation at 30°C was completely inhibited in all replicates at 40 µg ml^{-1} kanamycin. Transformant colonies obtained through transposon mutagenesis grew at kanamycin concentrations up to and including 100 µg ml^{-1} in all replicates.

Transformation

To date, we have obtained 593 putative transformant colonies from four independent transformation reactions. The first three transformations were achieved relatively late in the *V. spinosum* growth cycle (late log to early stationary phase), using 1 µl transposome, and typically produced ~10–50 transformant colonies per transformation attempt. The fourth transformation was achieved in early-to-mid log phase using 2 µl transposome and produced 492 putative transformant colonies, corresponding to a transformation efficiency of ~12 cfu per ng transposome.

Confirmation and mapping of transposon insertions

We confirmed presence of Tn5 within the chromosomal DNA of a subset of 12 putative transformants via PCR. All transformant colonies tested produced a 1.2 kb amplification product (standard agarose gel electrophoresis; data not shown), thus confirming transposon insertion. Inverse PCR (iPCR) as described by Fernandes et al. (2001) was performed on a further 24 putative transformants to both verify transposon insertion and sequence the genomic DNA flanking the insertion sites. The iPCR products were examined via standard agarose gel electrophoresis and found to vary in size from 250 base pairs to greater than 1 kb (Fig. 1), indicating differing insertion sites for the transposon. Fourteen iPCR amplicons were sequenced to identify the genomic location of the transposons. Transposon locations included genes predicted to encode a sensor histidine kinase KdpD, serine/threonine kinase Pkn10, TetR family protein, and an ABC-type multidrug transport system (Table 1). Mapping of these insertions onto the *V. spinosum* genome (Online Resource 1) confirmed that most analyzed transposition events were randomly distributed within the genome, as suggested by the iPCR results described above.

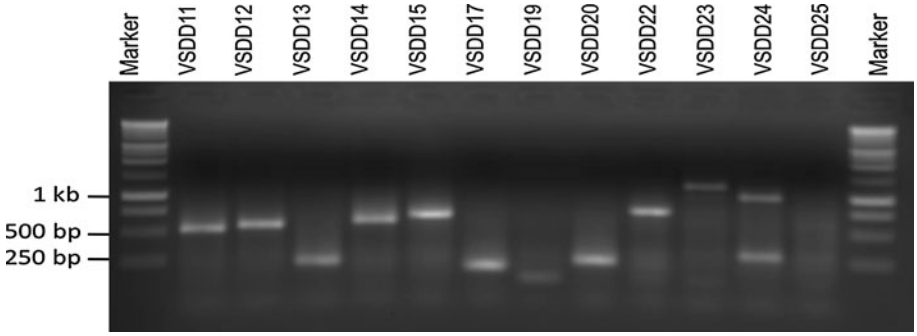

Fig. 1 Inverse PCR products of various sizes, obtained from 12 *V. spinosum* transformants. Some transformants, such as VSDD24, appear to have double insertions of the Tn5 transposon

Table 1 Predicted gene products of transposon mutants obtained from *V. spinosum*

Mutant	Position in genome (nt) [a]	Predicted gene product [b]
VSDD3	6864706	Hypothetical protein
VSDD5	3800971	Sensor histidine kinase KdpD
VSDD7	6773371	Intergenic region
VSDD511	8068053	Serine/threonine kinase Pkn10
VSDD12	1495590	Hypothetical protein
VSDD13	8007120	Hypothetical protein
VSDD14	6322558	Transcriptional regulator, TetR family protein
VSDD15	4152834	NAD-dependent protein deacetylases, SIR2 family
VSDD17	125559	Probable phosphoenolpyruvate-protein phosphotransferase
VSDD20	3817426	Intergenic region
VSDD21	5587756	ABC-type multidrug transport system, ATPase component
VSDD22	7083626	Outer membrane efflux protein
VSDD23	1912722	Glycosyl transferase family 2
VSDD52	5587874	ABC-type multidrug transport system, ATPase component

[a] Coordinate in the GenBank sequence accession ABIZ00000000

[b] Obtained from the Integrated Microbial Genome database (Joint Genome Institute)

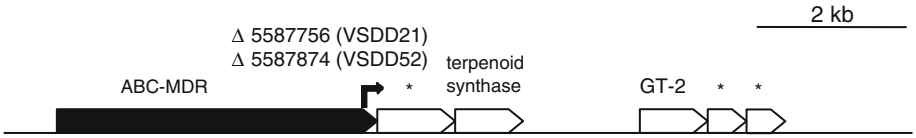

Fig. 2 Genetic map of VSDD21 and VSDD52 insertion sites and local gene organization. Both mutations occurred within a predicted ABC-type multidrug transport system (ABC-MDR). Hypothetical proteins are denoted with '*Asterisk*', and the putative glycosyl transferase family 2 gene is indicated by 'GT-2'

Phenotypic screening

To identify phenotypes associated with transposon mutagenesis, we screened 101 transformants (VSDD1-VSDD101) for the ability to metabolize glucose as the sole carbon source on VL70 minimal medium (Schoenborn et al. 2004). Two transformants, VSDD21 and VSDD52, failed to grow on VL70, but demonstrated growth on M13 agar, which contains peptone. Sequencing of iPCR products from these transformants revealed insertion within the same ABC-type multidrug transport system (ATPase component) gene. The insertion of VSDD21 occurred 116 bp downstream of VSDD52, at the 3′ terminal end of the gene as seen in Fig. 2. It does not appear that the inability to utilize glucose has been previously associated with mutations in ABC-type multidrug transport systems. Since both mutations occurred at the 3′ end of the gene (Fig. 2), these phenotypes may result from polar effects on downstream genes, with the most likely candidate being the predicted glycosyl transferase family 2 (GT-2) gene (Campbell et al. 1997). Systematic disruption of the three genes constituting this gene cluster would allow us to definitively test the hypothesis that polar effects on downstream genes contribute to this observed phenotype.

Development of genetic tools for the Verrucomicrobia

To our knowledge, this study represents the first reported successful transformation of a member of the Verrucomicrobia. Further optimization to obtain higher transformation efficiencies will be necessary for the generation of large libraries. This work is a useful first step in the development of genetic tools for further study of *V. spinosum*, e.g., testing the role of predicted type III secretion systems in eukaryotic interactions (Pallen et al. 2005) or identification of genes associated with the distinctive Planctomycete–Verrucomicrobial cell plan (Lee et al. 2009). The glucose mutants could also be further investigated. The random transposon mutagenesis approach described here could also be tested on other Verrucomicrobial strains, and if successful would allow elucidation of the genetic basis for Verrucomicrobial properties of interest.

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