Analyses of the *vrl* Gene Cluster in *Desulfococcus multivorans*: Homologous to the Virulence-Associated Locus of the Ovine Footrot Pathogen *Dichelobacter nodosus* Strain A198

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Abstract

Major parts of the virulence-associated *vrl* locus known from the gammaproteobacterium *Dichelobacter nodosus*, the causative agent of ovine footrot, were analyzed in the genome of the sulfate-reducing deltaproteobacterium *Desulfococcus multivorans*. In the genome of *D. multivorans* 13 of the 19 *vrl* genes described for *D. nodosus* are present and highly conserved with respect to gene sequence and order. The *vrl* locus and its flanking regions suggest a bacteriophage-mediated transfer into the genome of *D. multivorans*. Comparative analysis of the deduced Vrl proteins reveals a wide distribution of parts of the virulence-associated *vrl* locus in distantly related bacteria. Horizontal transfer is suggested as driving mechanism for the circulation of the *vrl* genes in bacteria. Except for the *vrlBMN* genes *D. multivorans* and *Desulfovibrio desulfuricans* G20 together contain all *vrl* genes displaying a high degree of similarity. For *D. multivorans* it could be shown that guanine plus cytosine (GC) content, GC skew, di-, tri- or tetranucleotide distribution did not differ between the *vrl* locus and its flanking sequences. This could be a hint that the *vrl* locus originated from a re-lated organism or at least a genome with similar characteristics. The conspicuous high degree of conservation of the analyzed *vrl* genes may result from a recent transfer event or reflect a function of the *vrl* genes, which is still unknown and not necessarily disease associated. The latter is supported by the evidence for expression of the *vrl* genes in *D. multivorans*, which has not been described as pathogen or to be associated to any disease pattern before.

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Introduction

The virulence-associated locus (*vrl* locus) has originally been described for *Dichelobacter nodosus*. This Gram-negative anaerobic rod-shaped bacterium belongs to the Gammaproteobacteria [Dewhirst et al., 1990; La Fontaine et al., 1990] and represents the essential causative agent of ovine footrot. Footrot is regarded as the most important bacterial disease of sheep, resulting in heavy economic losses in wool and meat industries. The occurrence of ovine footrot is widely distributed in temperate climates [Hindmarsh et al., 1985; Jimenez et al., 2004; Morck et al., 1994; Rood et al., 1996; Wani et al., 2004; Younan et al., 1999; Zakaria et al., 1998].

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The disease is characterized by a separation of the horn of the hoof from the underlying soft tissue, which leads to lameness and deterioration of body condition [Haring et al., 1995]. Strains of *D. nodosus* are classified as virulent (high/low), intermediate or benign, according to the severity of the disease. *D. nodosus* strain A198 exhibits potential virulence factors including type IV fimbriae and several extracellular proteases. Two genomic regions are preferentially associated with virulent isolates: the *vap* (virulence-associated proteins) regions and the *vrl* locus. The *vap* regions can be found in 95% of virulent and high intermediate isolates, and in 88% of the intermediate strains, but in only 38% of low intermediate and benign strains. The *vrl* sequences are present in 77% of virulent and high intermediate strains, but in only 13 and 7% of intermediate/low intermediate and benign strains, respectively. Thus, the *vrl* locus appears to be more specifically associated with *D. nodosus* isolates of greater virulence [Billington et al., 1996]. The *vrl* locus in *D. nodosus* strain A198 has a size of 27.1 kb, a higher G+C content than the chromosomal average, and contains 19 open reading frames (ORFs; designated *vrlA* through *vrlS*). Variants of the *vrl* locus characterized by deletions are known from other *D. nodosus* strains. All gene products are predicted to be localized in the cytoplasm. Only a few proteins encoded in the *vrl* locus were functionally characterized based on analyzing the deduced protein sequences and on expression studies [Billington et al., 1999]. In addition, functional prediction is limited by the fact that none of the *vrl* encoded proteins displays homology to known virulence factors.

D. nodosus strain A198 possibly acquired the *vrl* locus via insertion of a bacteriophage or conjugative plasmids, since (1) the G+C contents of the *vrl* region and the chromosome differ (58% vs. 45%); (2) a putative bacteriophage-like attachment site at the 5′ end of the *vrl* locus, and (3) *vrl* genes encoding functions often associated with extrachromosomal elements are present [Billington et al., 1999; Haring et al., 1995]. Such a system could allow transfer of the *vrl* locus between distantly related bacteria [Cheetham et al., 1995].

Nevertheless, the presence of the *vrl* locus in *Desulfococcus multivorans* was surprising, since the latter has thus far not been described as a pathogen or disease-related. *D. multivorans* is a Gram-negative, sulfate-reducing bacterium belonging to the Deltaproteobacteria [Devereux et al., 1989]. It is a metabolically versatile bacterium capable of utilizing aromatic compounds by employing novel selenocysteine-containing proteins [Peters et al., 2004; Rabus et al., 2001] and producing poly-hydroxyalkanoates as storage compounds [Hai et al., 2004]. The bacterium is also reported to have an extracellular metal-binding activity possibly compensating the precipitation of metal ions caused by sulfide production [Bridge et al., 1999]. Highly conserved genes of the *vrl* locus were detected during a whole genome shotgun (WGS) sequencing of *D. multivorans*. We determined the sequence of this *vrl* locus (including flanking regions) and the expression of the predicted *vrl* genes. Comparison of the gene sequences and order in the *vrl* locus of *D. multivorans* with related genomes provided insights into gene variants, transfer mechanisms and possible origin of the *vrl* locus.

Results

Overview of the Studied DNA Fragment

Detection of orthologs of the *vrl* genes from *D. nodosus* motivated the determination of an 89,210-bp fragment of the *D. multivorans* genome. This contiguous sequence (contig) was reconstructed from two completely sequenced fosmids and 705 sequences obtained from WGS sequencing. A total of 78 ORFs were predicted on the assembled contig, 23 of which could be assigned to the *vrl* locus (fig. 1). InterPro/COG references, BLASTP hits and assigned functions for each ORF of the studied DNA fragment are provided in supplement 2 (www.karger.com/doi/10.1159/000103607). The *vrl* locus is framed by direct repeats (fig. 3), probably originating from an integration event. The genes of the upstream region are apparently not functionally related to the *vrl* locus. For example, *smpB*, located next to the *vrl* locus, codes for the RNA-binding protein essential for the peptide-tagging activity of SsrA (tmRNA) [Karzei et al., 1999]. The latter is encoded immediately downstream of *smpB*.

In *D. multivorans* the *vrl* locus includes the genes *vrlEFGHIJKL*, *dmi39* (coding for an ATPase), *dmi40* (coding for a hypothetical protein) and *vrlOPQRS*. Some of the upstream genes have probably been transferred along with the *vrl* genes into the *D. multivorans* genome: *dmi26* (a bacteriophage-related protein), *dmi27* (encoding a site-specific recombinase), *dmi28* (encoding a bacteriophage-related protein), and *dmi29* (encoding a putative hypothetical protein).

The present functional prediction agrees with that of Billington et al. [1999]. VrlG contains a glutaminase domain of glutamine amidotransferases. VrlI harbors a helix-turn-helix domain and probably represents a transcriptional regulator, which is necessary for the tran-
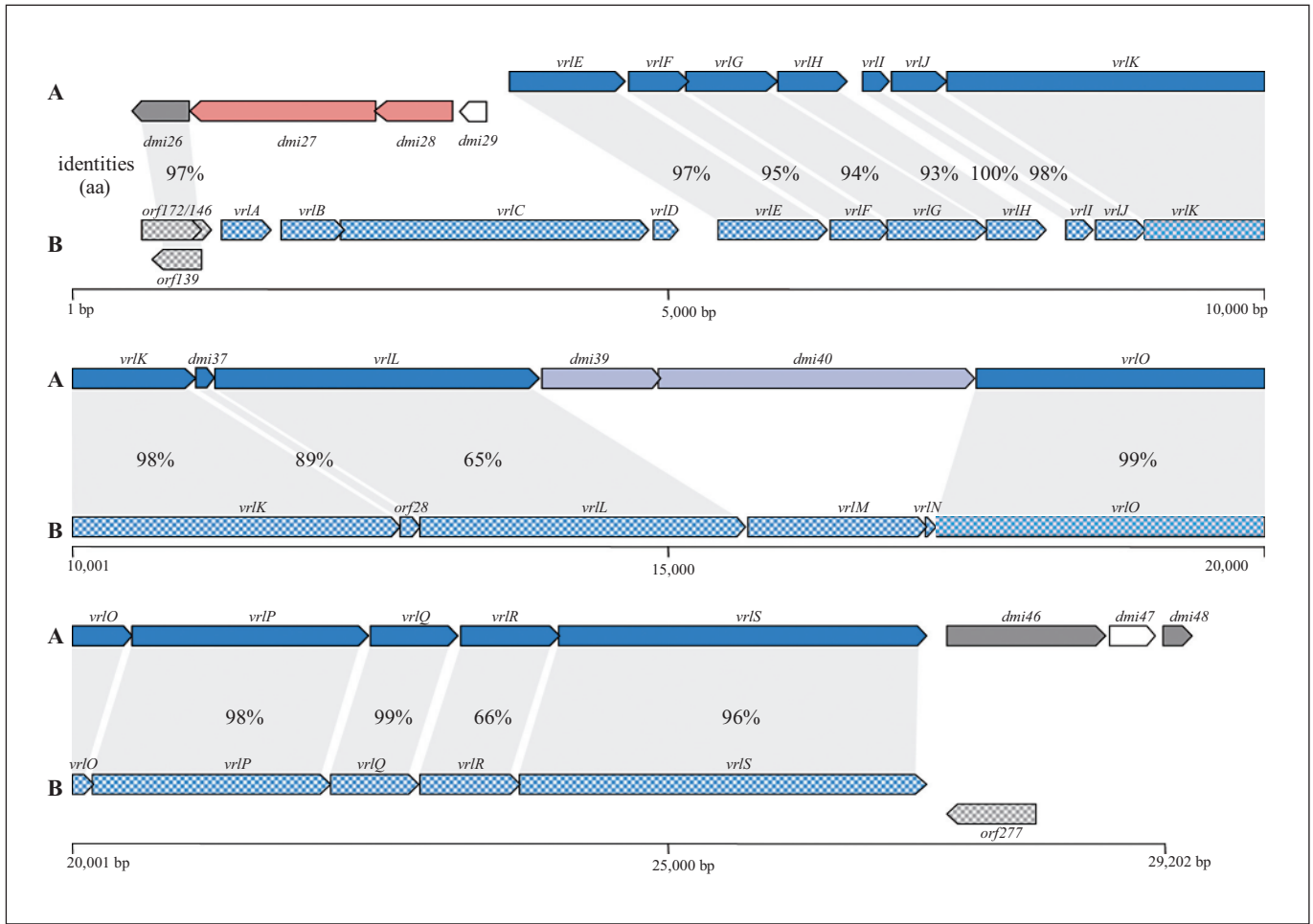
Fig. 1. Comparison of the *vrl* loci from *Desulfococcus multivorans* (**A**) and *Dichelobacter nodosus* str. A198 (**B**). Scale relates to nucleotide position in the known *D. nodosus* sequence.

scription of the other *vrl* genes in *D. nodosus* strain A198 [Billington et al., 1999]. A sigma 70 promoter is located in front of the *vrlI* gene in *D. nodosus* and *D. multivorans*. VrlJ contains an ATP binding motif. VrlK (contains an ATP- and a GTP-binding motif) and VrlP have weak similarities to PglY and PglZ, respectively, from *Streptococcus coelicolor*. VrlL represents a putative N6-adenine methyltransferase, while VrlO and VrlS are probably DEAH ATP-dependent helicases. The *vrl* genes are followed by three potential genes *(dmi46* through *dmi48)*, which code for proteins of unknown functions and are probably part of the *vrl* locus.

Genes further downstream of the *vrl* locus code for a type I restriction modification system *(dmi51, dmi52, dmi54)* [Chaturvedi and Chakravorty, 2003; Kita et al., 2003], a predicted virulence protein *(dmi57)* [Blanc-Po-

tard et al., 1999] and two ORFs representing fragments of an IS-element *(dmi60* and *dmi61)*, probably also resulting from transfer events.

Comparative Analysis of the vrl *loci of* D. multivorans *and* D. nodosus *Strain A198*

The orthologous products of the *vrl* genes from *D. multivorans* and *D. nodosus* share a high degree of sequence similarity (average 92%). The *vrl* locus in *D. multivorans* (fig. 1) starts with *dmi26* (coding for a hypothetical protein), which is homologous to *orf139* (AAC33376) in *D. nodosus* strain A198. Surprisingly, *vrlABCD* of *D. nodosus* are absent in *D. multivorans*. Instead, genes coding for a site-specific recombinase *(dmi27)*, a bacteriophage-related protein *(dmi28)* and a hypothetical protein *(dmi29)*, possibly representing parts of a phage transfer
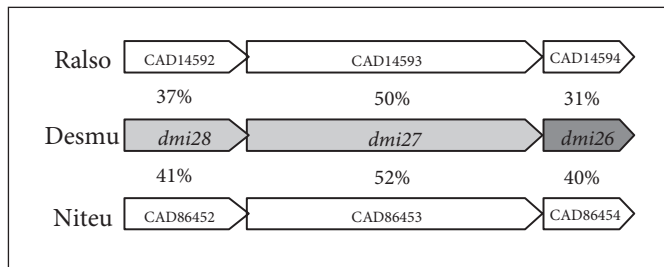
Fig. 2. Orthologs of the *dmi26/27/28* genes from *Desulfococcus multivorans* (Desmu) in *Ralstonia solanacearum* (Ralso) and *Nitrosomonas europaea* ATCC 19718 (Niteu). The identities (aa) from BESTFIT alignment are shown.

system are present. These genes are orientated in opposite direction, as compared to the subsequent *vrl* genes in *D. multivorans* and *vrlABCD* genes in *D. nodosus*, respectively. The genes *dmi26/27/28* in *D. multivorans* are present in the same order also in the genomes of *Ralstonia solanacearum* and *Nitrosomonas europaea* ATCC 19718 (fig. 2), even though the *vrl* locus is absent in both of them. Despite their overall homology, the N-terminal regions of Dmi26 from *D. multivorans* and Orf139 from *D. nodosus* strain A198 (supplement 3; www.karger.com/doi/10.1159/000103607) share no sequence similarity.

Different gene compositions of the *vrl* locus are known among *D. nodosus* strains. While the *vrl* locus in *D. nodosus* strain A198 consists of the *vrlABCDEFGHIJKLMNOPQRS* genes, it is also known that the *vrlOPQRS* genes in combination with the *vap* region are sufficient for virulence of *D. nodosus* [Billington et al., 1999]. Notably, the *vrlOPQRS* genes are present in *D. multivorans*.

The *vrl* genes of *D. multivorans* are highly similar to their counterparts in *D. nodosus* strain A198 (fig. 1). Sequence comparisons on nucleotide and amino acid level (BESTFIT alignments) show an average identity of 92%. In addition, *dmi26*, *vrlEFGHIJKL* and *vrlOPQRS* show the same gene order and orientation.

Instead of the *vrlMN* genes *(D. nodosus)* the *dmi39* and *dmi40* genes are present in *D. multivorans*. Their orthologs in *Bordetella bronchiseptica* (CAE31409, CAE31410) have the same orientation and are located next to each other on the upstream side by genes coding for an integrase (CAE31405) and a helicase (CAE31407). The latter indicates the possibility of a mobilization of this unit.

Homology between the *vrl* loci of *D. multivorans* and *D. nodosus* strain A198 ends beyond *vrlS*, the last gene of the *vrl* locus in *D. nodosus*. The up- and downstream parts of the *vrl* locus of *D. nodosus* are currently unknown

[Billington et al., 1999]. However, further analysis will be possible, when the complete genome sequence of *D. nodosus* VCS1703A (http://www.tigr.org) becomes available.

Traces of a Horizontal Transfer Event
The *vrl* locus was previously suggested to be acquired by horizontal transfer in *D. nodosus* strain A198 [Haring et al., 1995]. In case of *D. multivorans* such a horizontal transfer appears likely, considering on the one hand the high degree of conservation in gene sequence and organization compared to *D. nodosus* and on the other hand the phylogenetic distance contrasting this observation.

The *vrl* locus and the genome of *D. multivorans* display similar guanine plus cytosine (GC) contents (57.4% vs. 56.4%). In contrast, the *vrl* locus of *D. nodosus* shows a considerably higher GC content (58%; U20246) than the chromosome (45%) [Billington et al., 1999]. Such deviating GC contents are one typical indication for horizontally transferred genetic elements [Hacker and Kaper, 2000]. To investigate further hints for a horizontal transfer event GC content, GC skew, di-, tri- and tetranucleotide distribution of the *D. multivorans* contig were analyzed (data not shown). Results did not reveal any deviations which would be indicative of such an event. It should be noted that the absence of deviations indicate a genetic relatedness of the *vrl* locus and the genome of *D. multivorans*.

However, it was possible to identify the suggested bacteriophage-like attachment site *(attL)* [Billington et al., 1999] at the 5′ end of the *vrl* locus. This *attL* site is partially located within a direct repeat flanking the *vrl* locus in *D. multivorans*. We propose that these perfect 30mer direct repeats (fig. 3) represent the borders of the *vrl* locus and that they resulted from an integration event and partially overlap with 3′ end of *ssrA*, a typical integration point for prophages in bacterial genomes and therefore an indicator for past horizontal transfer events [Pelludat et al., 2003; Williams, 2003].

Circulation of the vrl *Genes*
The *vrl* genes were originally defined in *D. nodosus* [Haring et al., 1995]. Orthologous proteins for parts of the *vrl* cluster could be identified in several other genomes (table 1; supplement 5; www.karger.com/doi/10.1159/000103607). The upper part containing *vrlACDEFGHI* could be identified for *D. desulfuricans* G20 (two copies) and *Syntrophobacter fumaroxidans* MPOB. The absence of a *vrlB* gene could be explained by closely examining the *vrlAC* gene products. The deduced proteins
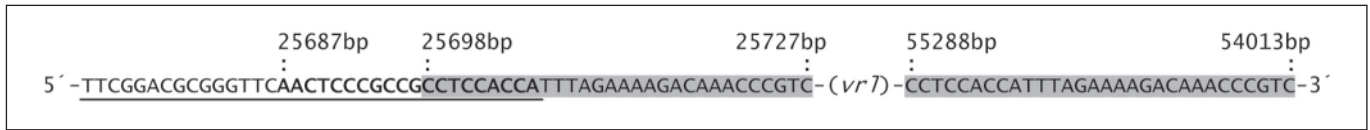
```
          25687bp      25698bp              25727bp    55288bp            54013bp

5´-TTCGGACGCGGGTTCAACTCCCGCCGCCTCCACCATTTAGAAAAGACAAACCCGTC-(vrl)-CCTCCACCATTTAGAAAAGACAAACCCGTC-3´
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Fig. 3. Flanking regions of the *vrl* locus in *Desulfococcus multivorans*. Positions of the direct repeats are highlighted in grey. The sequence homologous to the suggested phage attachment site *attL* of *D. nodosus* str. A198 is marked in bold letters. The 3′ end of the tmRNA is underlined. Nucleotide numbers refer to the position in the determined 89-kb DNA fragment of *D. multivorans*.

Table 1. Circulation of parts of *vrl* locus in the kingdom Bacteria[a]

Organisms with Vrl orthologs	Deduced Vrl proteins of the *vrl* locus in *D. nodosus*																		
	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S
Actinobacteria																			
Acidothermus cellulolyticus										●	●	●			●	●	●		
Bacteroidetes																			
Chlorobium phaeobacteroides[b]							●	●			●								
							●	●	●										
Firmicutes																			
Moorella thermoacetica											●	●			●	●			
Pelotomaculum thermopropionicum											●	●			●	●	●		
Thermoanaerobacter ethanolicus										●	●	●			●	●	●		
Gammaproteobacteria																			
Nitrosococcus mobilis										●	●	●			●	●	●		
Nitrosococcus oceani										●	●	●			●	●	●		
Deltaproteobacteria																			
Desulfococcus multivorans					●	●	●	●	●	●	●	●			●	●	●	●	●
Desulfovibrio desulfuricans[b]	○		○	●	●	●	●	●	●										
	○		○	●	●	●	●	●	●										
Syntrophobacter fumaroxidans	○		○	●	●	●	●	●	●										

[a] A filled circle marks deduced proteins in the different organism. Empty circles mark the orthologous parts of VrlAC, which are represented by a single gene in *D. desulfuricans* and *S. fumaroxidans*. For additional information see supplement 5 (www.karger.com/doi/10.1159/000103607).

[b] Two copies of *vrl* genes present in the genome.

VrlAC of *D. nodosus* are homologous to the N- and C-terminal part of single proteins in *D. desulfuricans* G20 (ABB37755) and *S. fumaroxidans* MPOB (EAO21197). One may speculate that *vrlB* was inserted into a primordial gene, the product of which contained VrlAC homologous domains. But it may also be true that *vrlAC* and *vrlB* originated from a single degenerated phage-related gene.

The largest number of orthologous genes (*vrlEFGHI-JKLOPQRS*) is present in *D. multivorans*. Smaller fragments (table 1; supplement 5, www.karger.com/doi/10.1159/000103607) of the *vrl* locus occur in *Acidothermus cellulolyticus* 11B (*vrlJKLOPQ*), *Nitrosococcus mobilis* NB-231 (*vrlJKLOPQ*), *Nitrosococcus oceani* ATCC 19707 (*vrlJKLOPQ*), *Thermoanaerobacter ethanolicus* ATCC 33223 (*vrlJKLOPQ*) and *Pelotomaculum thermopropionicum* S1 (*vrlKLOPQ*). At least three clustered genes of the *vrl* locus are probably present in *Chlorobium phaeobacteroides* BS1 (*vrlGHK, vrlGHI*) and *Moorella thermoacetica* ATCC 391073 (*vrlKLOP*). The *vrlBMN* genes could only be detected in *D. nodosus* so far. In summary, parts of the *vrl* locus are widely distributed and could be found in Actinobacteria, Firmicutes, Gammaproteobacteria and Deltaproteobacteria. Highest similarities to the *vrl* locus could be identified in Deltaproteobacteria.
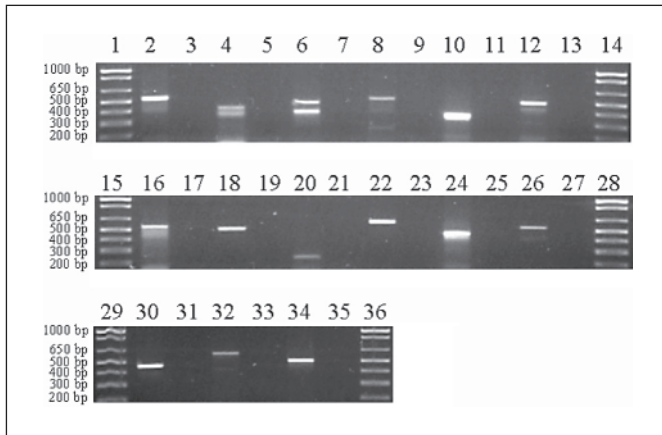
Fig. 4. RT-PCR analysis of ORFs and intergenic regions in the *vrl* locus. Lanes 1, 14, 15, 28, 29 and 36 size-marker; lane 2, product of the intergenic region between *vrlI* and *vrlJ*; lane 4, product of *vrlJ*; lane 6, product of the intergenic region between *vrlJ* and *vrlK*; lane 8, product of *vrlK*; lane 10, product of the intergenic region between *vrlK* and *vrlL*; lane 12, product of *vrlL*; lane 16, product of *dmi39*; lane 18, product of *dmi40*; lane 20, product of the intergenic region between *dmi40* and *vrlO*; lane 22, product of *vrlO*; lane 24, product of *vrlP*; lane 26, product of *vrlQ*; lane 30, product of the intergenic region between *vrlQ* and *vrlR*; lane 32, product of *vrlR*; lane 34, product *vrlS*. Lanes 3, 5, 7, 9, 11, 13, 17, 19, 21, 23, 25, 27, 31, 33 and 35: corresponding controls in which reverse transcriptase was absent from the reaction mixtures.

Interestingly, the *vrl* locus is absent in the genomes of the deltaproteobacterial sulfate-reducers *Desulfovibrio vulgaris* [Heidelberg et al., 2004] and *Desulfotalea psychrophila* [Rabus et al., 2004], the deltaproteobacterial sulphur reducer *Geobacter sulfurreducens* [Methe et al., 2003] and the archaeal sulfate reducer *Archaeoglobus fulgidus* [Klenk et al., 1997].

The results indicate that (1) the occurrence of the *vrl* locus is not limited to *D. nodosus*; (2) except *vrlBMN* all *vrl* genes occur in some deltaproteobacteria within overlapping fragments, and (3) the mechanism of transfer is putatively phage based.

Expression of vrl *Genes in* D. multivorans

The expression of genes of the *D. multivorans vrl* locus was studied with reverse transcriptase-polymerase chain reaction (RT-PCR) (fig. 4). RT-PCR products of expected length (supplement 1, www.karger.com/doi/10.1159/000103607) could be detected and subsequently identified by sequencing for *vrlJKL*, *dmi39/40* and *vrlOPQRS*, but not for *dmi26/27* and *vrlEFGH*. The genes *vrlI* and *dmi37* were not tested, since their length is too short

to design suitable primers. Intergenic regions between non-overlapping ORFs were also tested for read-through using RT-PCR. For this purpose primer pairs covering the intergenic region were used with the forward primer binding to the end of the upstream located ORF and the reverse primer binding to the beginning of the downstream located ORF. Products of expected length for the intergenic regions between *vrlI* and *vrlJ*, *vrlJ* and *vrlK*, *vrlK* and *vrlL*, *dmi40* and *vrlO*, and *vrlQ* and *vrlR* were detected and also identified by sequencing. The presence of a transcript covering the intergenic region between *vrlI* and *vrlJ* indicates that both genes should also be transcribed. The same is to be expected for *dmi37*, since it is part of the identified RT-PCR product covering the region between *vrlK* and *vrlL*. Expression of the *vrl* genes is probably controlled by a sigma 70 promotor, which is also present upstream of *vrlI* in *D. nodosus* (U20246) (supplement 4, www.karger.com/doi/10.1159/000103607).

The expression of *vrlI* through *vrlS* and the intergenic regions between the non-overlapping ORFs suggest that these genes are organized in an operon structure, which was previously also suggested for *D. nodosus* [Billington et al., 1999].

Discussion

The horizontal transfer of the *vrl* locus is driven by a bacteriophage integration event at the *ssrA* gene of *D. multivorans* similar to the integration event in *D. nodosus*. Since resulting deviations in the nucleotide distribution in the *vrl* locus of *D. multivorans* are not obvious, one may speculate that the origin of the *vrl* locus could be within an organism related to *D. multivorans* or at least a genome showing similar characteristics. The complete backbone of the *vrl* locus is already represented by the combined overlapping fragments in the sulfate-reducing deltaproteobacteria *D. multivorans (vrlEFGHIJKL* and *vrlOPQRS)* and *D. desulfuricans (vrlACDEFGHI)* (table 1). Therefore, one may even expect to find a continuous *vrl* locus in a related genome in future. The genes v*rlB* and *vrlMN* are excluded from this assumption, since they probably originated from recent insertion events.

Despite its association with virulence, the actual function of the *vrl* locus remains unclear. Since *D. nodosus* cannot be genetically manipulated so far, the relationship between this locus and virulence could not be further analyzed. Therefore the *vrl* locus cannot be unambiguously designated as a pathogenicity island (PAI) at present [Hacker and Kaper, 2000]. In this respect the occur-

rence of the *vrl* locus in *D. multivorans* is interesting, since this organism is thus far not associated with any disease. Moreover, there is no evidence for the presence of other virulence associated genetic elements of *D. nodosus* in the WGS data (4.4-fold sequencing coverage) of *D. multivorans*. The absence of *vap* genes is particularly striking, considering that for more than 800 *D. nodosus* isolates the presence of the *vrl* locus is coupled to that of the *vap* region [Rood et al., 1996].

The genes of the *vrl* locus in *D. multivorans* may serve a function, which is distinct from pathogenicity. The proven gene expression in *D. multivorans* is a hint for a possible functional activity. It is also conspicuous that the respective genes display an extraordinarily high degree of conservation. This conservation could be the result from a recent transfer event, but there is also the possibility of an unknown functional, not disease-related activity.

Materials and Methods

Bacterial Strain and Growth Conditions
D. multivorans (DSM 2059) was cultivated and cells were harvested as previously described [Collins et al., 1986; Hai et al., 2004].

Library Construction and DNA Sequencing
Total DNA was isolated using the Qiagen Genomic tip 500 system (Qiagen, Hilden, Germany). For WGS sequencing the isolated DNA was fragmented by sonication. Resulting fragments were end-repaired with T4 and Klenow polymerase (New England Biolabs, Beverly, Mass., USA), size selected, ligated in pUC19 vectors, transformed into *E. coli* DH10B (Invitrogen, Carlsbad, Calif., USA), and selected based on ampicillin resistance [Sambrook and Russel, 2001]. Two plasmid libraries with 1.3- and 3-kb inserts were obtained. These inserts were amplified by PCR [Radelof et al., 1998] as templates for sequencing with Big Dye Terminator chemistry and ABI 3730XL capillary sequencers (Applied Biosystems, Foster City, Calif., USA). The sequencing coverage from shotgun reads was about 4.4-fold. Additionally a copy control fosmid library (Epicentre, Madison, Wisc., USA) was constructed according to the manufacturer's instructions. A total of 192 fosmid clones, representing a physical genome coverage of at least 1-fold, were sequenced from both ends. Two fosmids were selected to extend WGS sequences containing *vrl*-genes, and sequenced by random integration approach using the KAN-2 Transposon Insertion Kit (Epicentre, Madison, Wisc., USA).

Sequence Assembly, Gene Prediction, Functional Assignment and Nucleotide Distribution
The quality of raw sequence data was determined with PHRED [Ewing et al., 1998; Ewing and Green, 1998]. Sequences were assembled with phrap2gap (http://www.sanger.ac.uk/Software/sequencing/docs/phrap2gap/) using PHRAP [Rieder et al., 1998] and finished in GAP4 [Staden et al., 2000], reaching a quality of less than one error in 20,000 bases.

GLIMMER2 [Delcher et al., 1999] was used for the prediction of genes on the finished 89 kb contig. The program was adjusted, falsely predicted ORFs were removed and ORFs refined as previously described [Rabus et al., 2002].

Similarity searches were carried out using the BLAST programs [Altschul et al., 1997] and by screening of the translated amino acid sequences against the NCBI database (http://www.ncbi.nlm.nih.gov). The predicted gene products were functionally assigned with the INTERPRO system [Apweiler et al., 2001] and screened against the Cluster of Orthologous Groups of Proteins [Tatusov et al., 2001].

Annotation of the identified ORFs was accomplished by ARTEMIS (http://www.sanger.ac.uk/Software/Artemis/). Alignments for nucleotide and amino acid sequences were generated with BESTFIT (Wisconsin Package Version 10.2, Genetics Computer Group (GCG), Madison, Wisc., USA), and used to determine identities. The annotated sequence has been deposited in Genbank/EMBL/DDBJ under accession number CT009609.

The determined complete sequence of the *vrl* locus and the deduced proteins of *D. nodosus* strain A198 (U20246) were used for comparative analysis based on BLASTP [Altschul et al., 1997] against the NCBI database (http://www.ncbi.nlm.nih.gov) to estimate the distribution and organization of the *vrl* genes in other organism. Analysis was limited to DNA fragments, which contain at least three proteins of the *vrl* locus in a genetic context in the examined sequence.

GC-content and GC-skew were analyzed using the program ARTEMIS (http://www.sanger.ac.uk/Software/Artemis/). The program SWAAP (http://www.bacteriamuseum.org/SWAAP/SwaapPage.htm) was used for determination of di-, tri- and tetranucleotide distributions.

RT-PCR Experiments
Total RNA was prepared from *D. multivorans* cells using the hot-phenol method as previously described [Aiba et al., 1981]. The dried RNA was dissolved in water, and potentially contaminating DNA was removed by treatment with RNase-free DNase (10 U μl^{-1}) (Roche, Basel, CH), 10× DNase buffer [Weinbauer et al., 2002], RnaseOut (Invitrogen, Carlsbad, Calif., USA) and 0.1 M DTT (Sigma, St. Louis, Mo., USA). cDNA was synthesized with gene specific reverse primers (MWG Biotech, Ebersberg, Germany) (supplement 1, www.karger.com/doi/10.1159/000103607) using the SuperScript First-Strand Synthesis System (Invitrogen, Carlsbad, Calif., USA) according to the manufacturer's protocol. PCR was carried out in a 100-μl scale under standard conditions using the synthesized cDNA as templates. To prove the absence of genomic DNA in the RNA preparations, controls (cDNA synthesis) were carried out without reverse transcriptase. Unambiguous identification of PCR products was based on sequencing.

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