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Analysis of the Heat Shock Response of *Neisseria meningitidis* with cDNA- and Oligonucleotide-Based DNA Microarrays

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Oligonucleotide- and cDNA-based microarrays comprising a subset of *Neisseria meningitidis* genes were assessed for study of the meningococcal heat shock response and found to be highly suitable for transcriptional profiling of *N. meningitidis*. Employing oligonucleotide arrays encompassing the entire genome of *N. meningitidis*, we analyzed the meningococcal heat shock response on a global scale and identified 55 heat shock-deregulated open reading frames (34 induced and 21 repressed).

Sequencing of the genomes of *Neisseria meningitidis* serogroup A and serogroup B strains provided us with a tremendously broad range of information (15, 23). The next step is the elucidation of gene expression patterns and gene product function on a genome-wide scale. DNA microarrays offer an ideal tool for high-throughput investigation of gene regulation on the transcriptional level (for review, see references 4, 13, and 17). The two most commonly employed DNA microarray platforms are oligonucleotide and cDNA arrays. Here we performed a comparative analysis of the suitability of both technology platforms for transcriptional profiling of *N. meningitidis*.

The two main features of DNA microarray performance are sensitivity (signal intensity) and specificity (ratio of specific to nonspecific hybridization). Additional care must be taken to standardize experimental conditions and to avoid the detection of false-positive signals (12). In order to validate gene expression modulations of *N. meningitidis* observed using cDNA-based and oligonucleotide-based microarrays, we first performed parallel hybridizations of identical RNA samples to the same slide. Microarrays containing probes specific for 60 genes selected from the published genome sequence of *N. meningitidis* serogroup B strain MC58 (23) were produced (Table 1). For cDNA-arrays, internal fragments of each open reading frame (ORF) (300 to 560 bp) were PCR amplified. For oligonucleotide arrays, oligonucleotides (40-mers, three per gene) comprising gene-specific internal fragments (covering 5′, central, and 3′ parts) were designed. All oligonucleotides (manufactured by MWG-Biotech AG, Ebersberg, Germany) carried a C6 amino linker modification at the 5′ end for covalent attachment to the slide surface. Each probe was spotted 5 (oligonucleotides) or 10 times (PCR products) per array using the Affymetrix 417 Arrayer (MWG-Biotech AG). PCR products were spotted on CMT-GAPS-Coated Slides (Corning, Wiesbaden, Germany), oligonucleotides were spotted on Super Aldehyde Slides (TeleChem International, Sunnyvale, Calif.), and the slides were processed according to the manufacturers' instructions.

Cultures of *N. meningitidis* strain MC58 (24) were grown to mid-logarithmic growth phase (optical density at 600 nm [OD_{600}] = 0.5/5 × 10^8 CFU/ml) at 37°C in supplemented proteose peptone medium and RNA isolated as previously described (5). The RNA was split into two aliquots, and one-half was labeled with Cy3-dCTP, the other with Cy5-dCTP (Amersham Pharmacia, Freiburg, Germany) during a first-strand reverse transcription (RT) reaction using Superscript II RNase H^- reverse transcriptase (Life Technologies, Karlsruhe, Germany) and a balanced mixture (1 pmol each) of C-terminal primers specific for all genes present on the microarrays. Before performance of the RT reaction, four different *Saccharomyces*-specific in vitro-derived transcripts (CHS1, CHS2, GAS1, and FKS1) were added as internal standards to the two labeling reaction mixtures in defined ratios. The two differentially labeled cDNA samples were mixed and again split in halves for hybridization to a cDNA array and an oligonucleotide array under identical conditions (3× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate], 0.1% [wt/vol] sodium dodecyl sulfate [SDS], 50°C) for 16 h. Arrays were washed and scanned using the Affymetrix 418 Scanner (MWG-Biotech AG). Average signal intensity and local background measurements were obtained for each spot using ImaGene 4.0 software (Biodiscovery Inc., Los Angeles, Calif.). The two channels were normalized with respect to the mean values of all *N. meningitidis* DNA spots. The Cy3/Cy5 fluorescence ratios were calculated from the normalized values, and the average signal ratios of all replicates per gene were determined (Fig. 1). Under identical conditions, all probes present should exhibit signals at a 1:1 ratio. On the cDNA arrays, the majority of genes (91%) were present at ratios ranging from 0.8 to 1.3, and five ORFs were present at ratios of 1.4 or 1.5 (Fig. 1A). Utilizing oligonucleotide arrays, 88% of the ORFs were present at ratios of 0.8 to 1.3, and seven ORFs were present at a ratio of

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TABLE 1. *N. meningitidis* serogroup B genes present on PCR product and oligonucleotide arrays comprising a subset of meningococcal ORFs

Loci[a] for:	Product (gene name)
Genes relevant under heat shock conditions	
NMB0027	FkbP-type peptidyl-prolyl *cis-trans* isomerase (*fbp*)
NMB0059	Heat shock protein (*dnaJ*)
NMB0550	Thiol/disulfide interchange protein (*dsbC*)
NMB0554	Heat shock protein 70 (*dnaK*)
NMB0791	Peptidyl-prolyl *cis-trans* isomerase
NMB1027	Truncation
NMB1131	Chaperone protein HscA (*hscA*-1)
NMB1313	Trigger factor (*tig*)
NMB1366	Thioredoxin
NMB1519	Thiol disulfide interchange protein (*dsbD*)
NMB1522	Peptidyl-prolyl *cis-trans* isomerase, FkbP-type (*slyD*)
NMB1649	Disulfide bond formation protein B (*dsbB*)
NMB1845	Thioredoxin
NMB1972	Heat shock protein (*groEL*)
NMB1973	Chaperonin (*groES*)
Competence/transformation genes	
NMB0018	Pilin, PilE (*pilE*)
NMB0049	PilC2 protein, authentic frameshift (*pilC2*)
NMB0116	DNA processing chain A (*dprA*)
NMB0118	DNA topoisomerase (*topA*)
NMB0269	Competence protein
NMB0329	Type IV pilus assembly protein (*pilF*)
NMB0332	Type IV prepilin peptidase (*pilD*)
NMB0405	Competence protein (*comM*)
NMB0692	Tpc protein (*tpc*)
NMB0702	Competence protein A (*comA*)
NMB0703	Competence protein L (*comL*)
NMB1445	RecA protein (*recA*)
NMB1588	CDP-diacylglycerol-glycerol-3-phosphate 3-phosphatidyltransferase (*pgsA*)
NMB1715	Multiple transferable resistance system protein MtrD (*mtrD*)
NMB1808	PilM protein (*pilM*)
NMB1809	PilN protein (*pilN*)
NMB1810	PilO protein (*pilO*)
NMB1811	PilP protein (*pilP*)
NMB1812	PilQ protein (*pilQ*)
NMB1847	PilC1 protein, authentic frameshift (*pilC1*)
NMB1936	ATP synthase F1 subunit (*atpA*)
NMB1940	ATP synthase F0 subunit (*atpB*)
NMB2044	Phosphoenolpyruvate protein phosphotransferase (*ptsI*)
Genes encoding restriction/modification systems	
NMB0829	Type I restriction enzyme *Eco*R124II M protein (*hsdM*)
NMB0831	Type I restriction enzyme S protein, degenerate
NMB0835	Type I restriction enzyme *Eco*R124II R protein
NMB1289	Type II restriction enzyme
NMB1375	Modification methylase, putative, authentic frameshift
Housekeeping genes	
NMB0207	Glyceraldehyde 3-phosphate dehydrogenase (*gapA*-1)
NMB0955	2-Oxoglutarate dehydrogenase, E1 component (*sucA*)
NMB1341	Pyruvate-dehydrogenase pdhA
NMB2159	Glyceraldehyde 3-phosphate dehydrogenase (*gapA*-2)
NMB0950	Succinate dehydrogenase, flavoprotein subunit (*sdhA*)
NMB0951	Succinate dehydrogenase, iron-sulfur protein (*sdhB*)
Sigma factors	
NMB1538	RNA polymerase sigma-70 factor (*rpoD*)
NMB0712	RNA polymerase sigma-32 factor (*rpoH*)
NMB2044	Sigma factor
NMB2144	Sigma factor
***nqr* operon**	
NMB0564	Na(+)-translocating NADH-quinone reductase, subunit F (*nqrF*)
NMB0565	Na(+)-translocating NADH-quinone reductase, subunit E (*nqrE*)
NMB0566	Na(+)-translocating NADH-quinone reductase, subunit D (*nqrD*)
NMB0567	Na(+)-translocating NADH-quinone reductase, subunit C (*nqrC*)
NMB0568	Na(+)-translocating NADH-quinone reductase, subunit B (*nqrB*)
NMB0569	Na(+)-translocating NADH-quinone reductase, subunit A (*nqrA*)
NO reductase	
NMB1622	Nitric oxide reductase (*norB*)

[a] ORF numbers in the published *N. meningitidis* serogroup B genome sequence (23) are given.

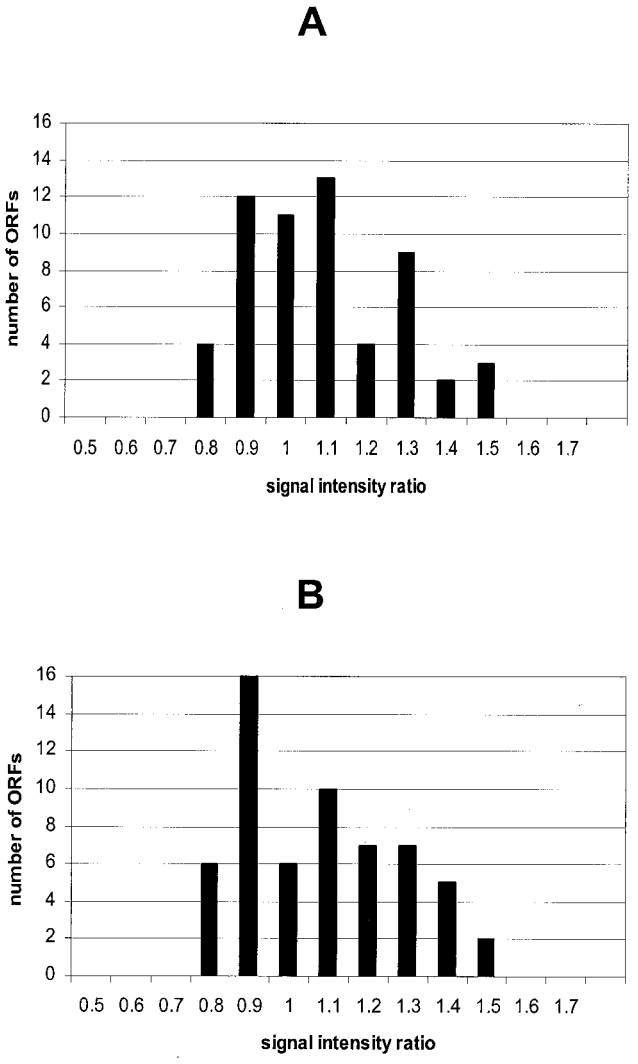

FIG. 1. Performance of cDNA-based versus oligonucleotide-based microarrays. RNA was isolated from a single *N. meningitidis* serogroup B culture, the RNA was split, and the halves were labeled independently. cDNAs were combined and split again for competitive hybridization with cDNA (A) and oligonucleotide arrays (B). After hybridization and washes, arrays were scanned and images were normalized. Histograms of average hybridization intensity ratios for individual ORFs are shown.

1.4 to 1.5 (Fig. 1B). The standard deviation was 0.19 for the PCR product arrays, and for oligonucleotide arrays, 0.20 for the combined three oligonucleotides per gene. Standard deviation and fluorescence intensity were inversely correlated for both array types. Signal intensity ratios of *Saccharomyces* internal standards reflected their ratios in the RNA samples (data not shown).

We next analyzed the standard deviation for RNAs isolated from different cultures of *N. meningitidis*. RNAs were isolated from cultures at mid-logarithmic growth phase (OD_{600} = 0.5) grown on different days. RNAs were labeled differentially and employed for competitive hybridization of cDNA microarrays (Fig. 2). The majority of ORFs exhibited signal ratios ranging from 0.8 to 1.2 (68%), some ORFs had a signal ratio of 0.7, and several had ratios of 1.3 to 1.6; the standard deviation was 0.21.

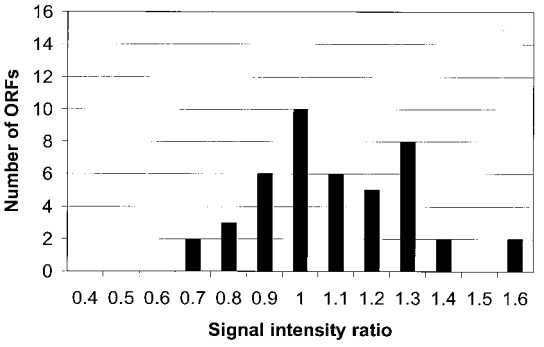

FIG. 2. Reproducibility of *N. meningitidis* cultures. RNAs were isolated from two neisserial cultures at mid-logarithmic growth phase (OD_{600} = 0.5) grown on different days. RNAs were labeled differentially and employed for competitive hybridization of cDNA arrays. A histogram of hybridization intensities for individual ORFs is shown.

The standard deviation of the microarray data is therefore mainly due to labeling and hybridization of RNAs; differences in culture conditions or RNA isolation have a minor impact. Based on these results, a 2-fold deregulation (equivalent to 3.29-fold $\log_2$ of the standard deviation of our experimental setting) has a level of confidence of above 99.9%, and a 1.7-fold deregulation (2.58-fold standard deviation) exhibits a confidence level of 99%. Usually, changes above 2-, 3-, and 4-fold variation are considered significant (25).

Sensitivity and specificity of PCR product and oligonucleotide arrays were compared for the analysis of the heat shock response of *N. meningitidis*. Meningococcal cultures were grown at 37°C to an OD_{600} of 0.5 and split into halves; one half was subjected to heat shock at 44°C for 5 min, and the other half remained at 37°C. RNA was isolated from both cultures 10 min post-heat shock. RNAs were labeled differentially, combined, and hybridized to cDNA and oligonucleotide microarrays. We found both array types to reproducibly detect ratios of transcript levels (Table 2). Eight of sixty ORFs were identified as being deregulated more than twofold by PCR product-based arrays; of these, six were also detected by oligonucleotide arrays. On the other hand, two ORFs were deregulated only according to the data analysis with oligonucleotide arrays. For the ORFs identified as deregulated by both array platforms,

TABLE 2. Analysis of *N. meningitidis* genes deregulated by heat shock at 44°C by PCR product and oligonucleotide arrays

Locus[a]/gene name	cDNA array		Oligonucleotide array	
	Transcript ratio	SD	Transcript ratio	SD
NMB0059/*dnaJ*	1.31	0.28	3.50	0.98
NMB0554/*dnaK*	3.77	0.19	1.79	1.12
NMB0565/*nqrE*	0.45	0.01	0.54	0.18
NMB0791/peptidyl-prolyl *cis-trans* isomerase	1.30	0.15	2.08	0.23
NMB1366/thioredoxin	3.80	0.12	4.18	1.03
NMB1810/*pilO*	0.39	0.02	0.51	0.05
NMB1936/*atpA*	0.56	0.14	0.49	0.00
NMB1940/*atpB*	0.50	0.02	0.89	0.08
NMB1972/*groEL*	2.76	0.13	3.91	1.39
NMB1973/*groES*	4.02	0.22	4.52	3.01

[a] ORF numbers in *N. meningitidis* serogroup B genome sequence (23).

the levels of deregulation were in excellent concordance. The majority of ORFs were detected by both platforms as being not deregulated (83%). Hence, there is a very good agreement of the data for oligonucleotide and cDNA microarrays. Similarly, cDNA and oligonucleotide arrays were recently found to exhibit a similar sensitivity and specificity for *Escherichia coli* (9) and *Saccharomyces* (8) transcriptome analysis.

RT-PCR was employed as an independent method to confirm the data obtained by microarray analysis for heat-shocked (44°C) versus untreated (37°C) meningococci. For *dnaK* (NMB0554), the increased transcript level was confirmed by RT-PCR. The RT-PCR assay also corroborated the microarray-based observation that the transcription of *pdhA* (NMB1341) was not changed by a shift to 44°C.

When analyzing the temperature dependence of the *N. meningitidis* heat shock response, we interestingly found only two ORFs to be deregulated more than twofold at 42 and 43.5°C, but 24 ORFs were deregulated more than twofold at 45°C (data not shown). While 45°C is certainly a nonphysiological condition for meningococci, the limited response at 42 and 43.5°C is in contrast to observations for *Neisseria gonorrhoeae*, where heat shock induction was observed on the RNA and protein level at 43°C (10, 22).

After the suitability of oligonucleotide and cDNA microarrays had been demonstrated, the meningococcal heat shock was analyzed for all 2,160 ORFs present in the genome of strain MC58 using oligonucleotide arrays manufactured according to the specifications given above. Three heat shock experiments were performed, comprising independent RNA isolations, labeling reactions, and hybridizations. Heat shock was performed at 45°C, and neisserial RNAs were isolated immediately after heat shock. Reference RNAs were isolated from parallel 37°C cultures. RNAs were labeled differentially, combined, and hybridized to whole-genome oligonucleotide microarrays. These arrays allowed hybridization signals specific for 1,498 different ORFs to be detected above the background level, equivalent to 69.35% of all MC58 ORFs. Data from the three independent experiments were combined, and average transcript ratios were calculated. Fifty-five ORFs (equivalent to 3.7% of the ORFs detected as being transcriptionally active) were identified as being heat shock responsive (34 upregulated, 21 downregulated; Table 3). This immediate deregulation after a shift to 45°C demonstrates the rapid onset of the heat shock response in *N. meningitidis*. The excellent reliability of the whole-genome microarrays for the analysis of the meningococcal transcriptome is demonstrated by the similar degree of deregulation found for genes organized in operons (NMB0164-0165, NMB0787-0788, NMB0906-0907, NMB0946-0947, NMB1468-1469, NMB1563-1564, NMB1789-1790, NMB1808-1812, and NMB1972-1973). The majority of meningococcal genes (95.9%) are not altered significantly due to heat shock, similar to the heat shock response of *E. coli* (18).

Most upregulated genes represent typical heat shock genes or are likely to be involved in the stress response, like those encoding chaperons and proteases, which protect stressed cells against protein misfolding. The heat shock sigma factor RpoH/σ32, the principal sigma factor RpoD/σ70, and a transcriptional regulator of the GntR family may be involved in the regulation of the heat shock response. Interestingly, two ORFs encoding ribosomal proteins were also identified as being up-

TABLE 3. *N. meningitidis* genes deregulated directly after heat shock at 45°C detected by whole genome arrays

Locus[a]	Gene name/product	Transcript ratio	SD
Genes upregulated			
NMB0059	*dnaJ*	2.90	1.10
NMB0554	*dnaK*	5.33	0.79
NMB0712	*rpoH*	1.63	0.48
NMB0791	Peptidyl-prolyl *cis-trans* isomerase	2.59	0.89
NMB1366	Thioredoxin	3.81	1.00
NMB1538	*rpoD*	3.06	1.08
NMB1972	*groEL*	3.70	0.59
NMB1973	*groES*	5.33	0.78
NMB0164	50S ribosomal protein L36	2.27	0.17
NMB0165	30S ribosomal protein S13	2.24	0.24
NMB0214	Oligopeptidase A	2.18	0.17
NMB0294	*dsbA*	2.48	1.17
NMB0370	Hypothetical protein	3.72	2.03
NMB0557	Conserved hypothetical protein	3.18	0.97
NMB0561	*grpE*	4.55	1.37
NMB0604	Alcohol dehydrogenase, zinc-containing	4.55	1.22
NMB0906	Hypothetical	2.50	0.43
NMB0907	Hypothetical	2.63	0.63
NMB0946	Peroxiredoxin 2 family Protein/glutaredoxin	4.18	1.38
NMB0947	Lipoamide dehydrogenase, putative	2.54	0.61
NMB1056	Hypothetical	4.60	2.10
NMB1231	ATP-dependent protease La	4.19	1.26
NMB1334	Hypothetical	2.41	0.32
NMB1377	L-lactate dehydrogenase	2.22	0.06
NMB1468	Hypothetical	2.68	0.51
NMB1469	Hypothetical	2.06	0.04
NMB1472	*clpB*	2.97	1.67
NMB1563	transcriptional regulator, GntR family	2.27	0.36
NMB1564	Conserved hypothetical protein	3.64	1.03
NMB1789	*secB*	2.34	0.20
NMB1790	Glutaredoxin 3	2.76	0.28
NMB1796	Conserved hypothetical protein	2.86	0.85
NMB2000	Conserved hypothetical protein	2.63	1.52
NMB2013	Hypothetical	2.37	0.56
Genes downregulated			
NMB0187	Ribosome recycling factor	0.62	0.25
NMB0535	Glucose/galactose transporter	0.63	0.26
NMB0565	*nqrE*	0.60	0.20
NMB0567	*nqrC*	0.78	0.13
NMB0568	*nqrB*	0.81	0.09
NMB0623	Spermidine/putrescine ABC Transporter periplasmic Spermidine/putrescine-binding protein	0.56	0.09
NMB0787	Amino acid ABC transporter, periplasmic amino acid-binding protein	0.38	0.26
NMB0788	Amino acid ABC transporter, permease protein	0.50	0.19
NMB0838	Cold-shock domain family protein	0.41	0.12
NMB0960	*sucD*	0.54	0.05
NMB1343	Hypothetical	0.56	0.04
NMB1669	Iron starvation	0.54	0.01
NMB1808	*pilM*	0.78	0.09
NMB1809	*pilN*	0.58	0.10
NMB1810	*pilO*	0.67	0.10
NMB1811	*pilP*	0.75	0.17
NMB1812	*pilQ*	0.52	0.06
NMB1933	*atpH*	0.58	0.09
NMB1936	*atpA*	0.78	0.08
NMB1937	*atpD*	0.61	0.11
NMB2051	*petC*	0.51	0.06

[a] ORF numbers in *N. meningitidis* serogroup B genome sequence (23).

regulated more than twofold. For 12 upregulated ORFs, no function has been identified so far.

The 21 downregulated ORFs are involved in aerobic metabolism and pilus synthesis and encode components of four different transporters, a cold shock protein, an iron starvation protein, and one ORF of unknown function. The exact mode of repression after heat shock in *N. meningitidis* is unclear. While upregulation is normally due to increased transcriptional activity, either downregulated ORFs may be transcribed at lower rates or the specific mRNAs may undergo more rapid degradation. For *E. coli*, the downregulation is not due to an increased decay of non-heat shock mRNAs (7). Although the heat shock response has been studied for many years, little has been reported about repressed genes. Here, the high proportion of ORFs involved in aerobic metabolism may indicate that their downregulation is caused by a decreased pO_2 of the culture medium at elevated temperatures. Similarly, in *E. coli* strains overexpressing recombinant proteins, a correlation of the induction of heat shock genes and the repression of genes involved in aerobic metabolism was demonstrated (14). The upregulation of the GntR family transcriptional regulator may play an important role in the neisserial heat shock response, since these regulators comprise repressors of genes involved in bacterial metabolism (19).

Our data demonstrate the physiological relevance of the microarray-determined transcriptional response of *N. meningitidis* to heat shock. The heat shock response is highly conserved among different organisms and allows cells to adapt rapidly to environmental and metabolic changes and to survive stress conditions. It is well studied among a wide range of microorganisms, with *Escherichia coli* being analyzed in the most detail (2). Whole-genome DNA arrays were recently applied to the analysis of the heat shock response for *E. coli* (18), group A *Streptococcus* (21), *Bacillus subtilis* (16), and *S. cerevisiae* (11). Among the *Neisseriae*, the heat shock response of *N. gonorrhoeae* has been elucidated on the protein (10, 26) and transcriptional levels (22). While increased levels of GroEL were found in cultures of *N. meningitidis* cultured under stationary growth conditions (1), this is the first analysis of gene regulation in *N. meningitidis* upon transient temperature increase.

Most microarray studies are performed with cDNA-based microarrays. However, oligonucleotide arrays offer an attractive alternative. The production of cDNA microarrays requires the PCR amplification of all genes to be included in the array. In addition, PCR products can have disadvantages, such as varying degrees of GC content and possible inclusion of sequence stretches with high homology to alternative ORFs, which causes nonspecific signal to interfere with the specific target sequence (3, 18). Regions sharing a homology above 75% of the length of oligonucleotide probes were recently shown to be sufficient for cross-hybridization (9). Oligonucleotides, in contrast, can be designed to the optimum, especially within a sequenced genome, enabling researchers to prevent nonspecific hybridization. Their specificity even allows the detection of single-nucleotide polymorphisms (6). Oligonucleotide arrays also allow the design of probes of minimal secondary structure and similar length and GC content. The 40-mer oligonucleotides chosen for this study were as sensitive and specific as PCR probes 300 to 560 bp in length. Recently microarrays using even 25-mer oligonucleotides were successfully employed for transcriptome analysis with *E. coli* (20).

In conclusion, our experimental data clearly demonstrate the suitability of oligonucleotide and cDNA microarrays for the study of gene regulation in *N. meningitidis*, and oligonucleotide-based whole-genome microarrays allowed the analysis of the *N. meningitidis* heat shock transcriptome.

M. Guckenberger and S. Kurz contributed equally to this work.

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