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**Publication Date**

2010-09-01





# Genetic Adaptation to Salt Stress in Experimental Evolution of *Desulfovibrio vulgaris* Hildenborough

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Ecosystems and Networks Integrated with Genes and Molecular Assemblies



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## INTRODUCTION

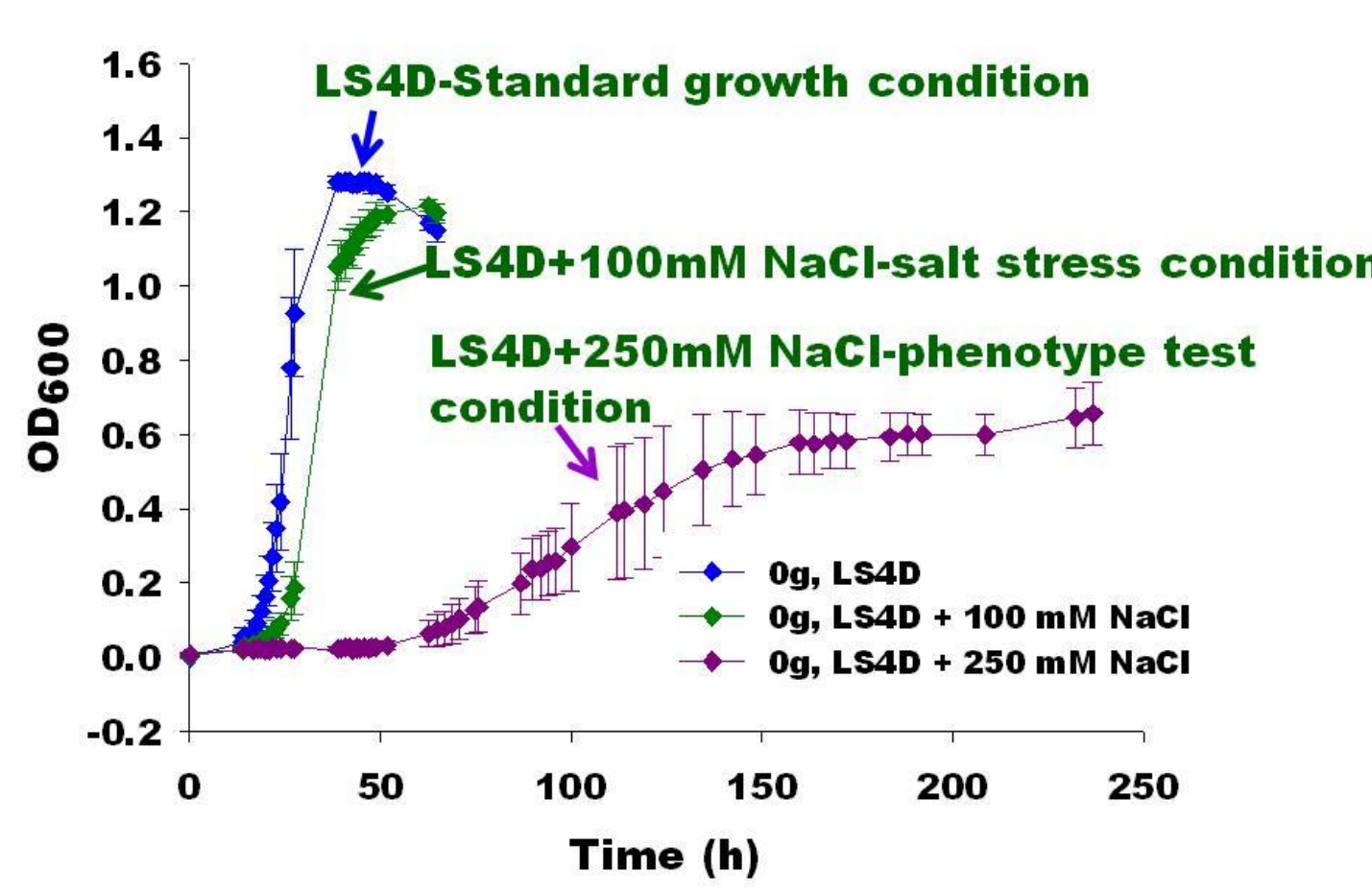
High salinity is one of the most common environmental stressors. In order to understand how environmental organisms adapt to salty environment, an experiment evolution with sulfate reducing bacteria *Desulfovibrio vulgaris* Hildenborough was conducted. Control lines and salt-stressed lines (6 lines each) grown in minimal medium LS4D or LS4D + 100 mM NaCl were transferred for 1200 generations. The salt tolerance was tested with LS4D supplemented with 250 mM NaCl. Statistical analysis of the growth data suggested that all lines adapted to their evolutionary environment. In addition, the control lines performed better than the ancestor with faster growth rate, higher biomass yield and shorter lag phase under salty environment they did not evolve in. However, the salt-adapted lines performed better than the control lines on measures of growth rate and yield under salty environment, suggesting that the salt-evolved lines acquired mutations specific to having extra salt in LS4D. Growth data and gene transcription data suggested that populations tended to improve till 1000 generations and active mutations tended to be fixed at the stage of 1000 generations. Point mutations and insertion/deletions were identified in isolated colonies from salt-adapted and control lines via whole genome sequencing. Glu, Gln and Ala appears to be the major osmoprotectant in evolved salt-stressed line. Ongoing studies are now characterizing the contribution of specific mutations identified in the salt-evolved *D. vulgaris*.

## MATERIALS AND METHODS

**Bacteria strain:** Single colony-based liquid culture was obtained from the original *D. vulgaris* Hildenborough stock. Six lines each were used for control and treatment respectively.  
**Medium and culture condition:** LS4D was used as standard medium for the control. Medium for salt stress treatment was LS4D +100 mM NaCl. Cells were kept at 37°C and transferred every 48 hrs with one to one hundred dilution.  
**Handling of the samples:** The glycerol stocks were archived for every 100 generations and a variety of molecular, physiological, and genomic analyses were conducted to monitor their evolution/adaptation to environmental stresses.  
**Microarray analysis:** 70mer oligonucleotide arrays for *D. vulgaris* Hildenborough that containing all ORFs (He et al., 2006) were used in this study. Total cellular RNA was isolated using TRIzol (Invitrogen) and RNeasy mini column and labeled with Cy5 dye. Genomic DNA was isolated from *D. vulgaris* Hildenborough as described previously (Zhou et al., 1996) and labeled with Cy3 dye. The labeled cDNA and genomic DNA were co-hybridized to the array. Microarray data were processed as described before (Chhabra et al., 2006; Mukhopadhyay et al., 2006).

## RESULTS

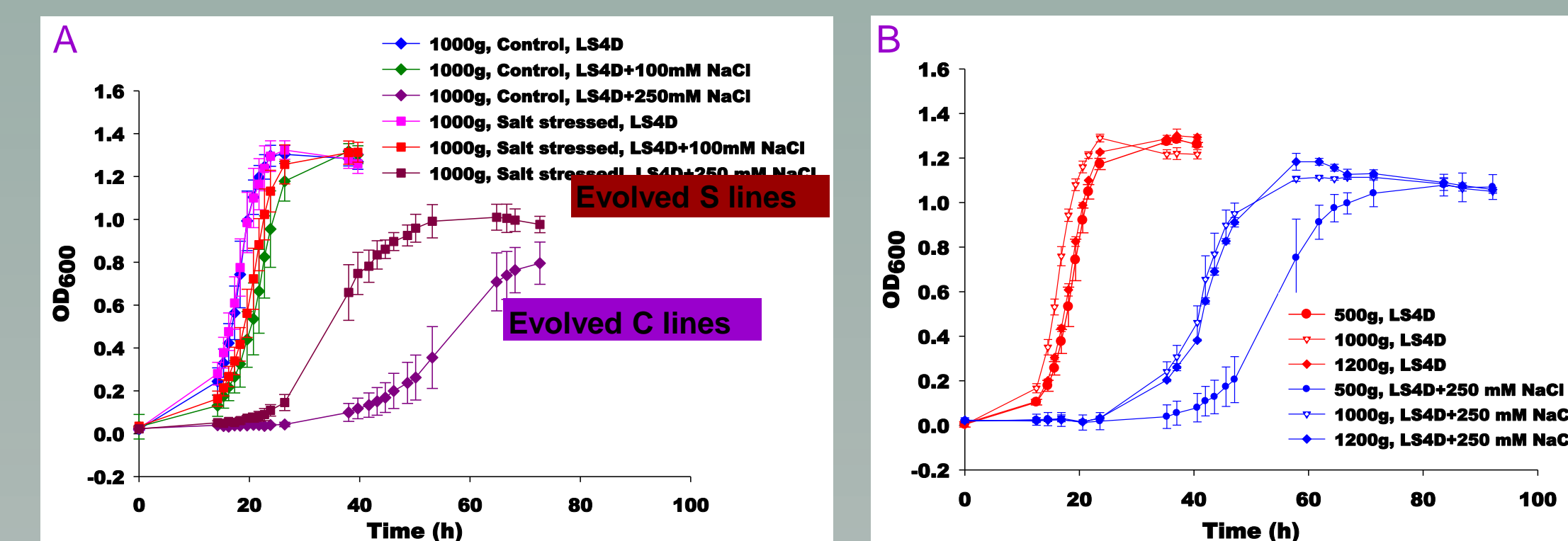
### Growth of *D. vulgaris* is arrested by elevated salt in the medium



With 100 mM NaCl in the medium, the growth rate and final biomass were not affected except a few hours longer of lag phase;  
With 250 mM NaCl in the medium, there was a very long lag phase and the final biomass was only half of the control.

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### Quick adaptation to salt during long-term evolution



A. Growth of the averages of six evolved control or salt stressed populations are shown. Shorter lag and higher biomass yield were observed for the evolved lines.  
B. The enhanced salt resistance phenotype appear to stabilize at 1000 generations. Growth of salt-stressed population #9 was shown.

### Adaptation to the evolution environment and dynamics of evolution

	LS4D			LS4D+250 mM NaCl		
	T vs A	C vs T	C vs A			
Growth rate	0.003778 (0.6054)	0.03589 (<0.001)	0.03967 (<0.001)	500g vs 1000g	500g vs 1200g	1000g vs 1200g
Yield	0.04528 (0.0787)	-0.01611 (0.5646)	0.02917 (0.2523)	-0.04033 (<0.001)	-0.05233 (<0.001)	-0.01200 (0.0926)
Lag	-6.6667 (0.0006)	-3.6889 (0.0772)	-10.3556 (<0.001)	-0.07833 (0.0168)	-0.06389 (0.0459)	0.01444 (0.6356)

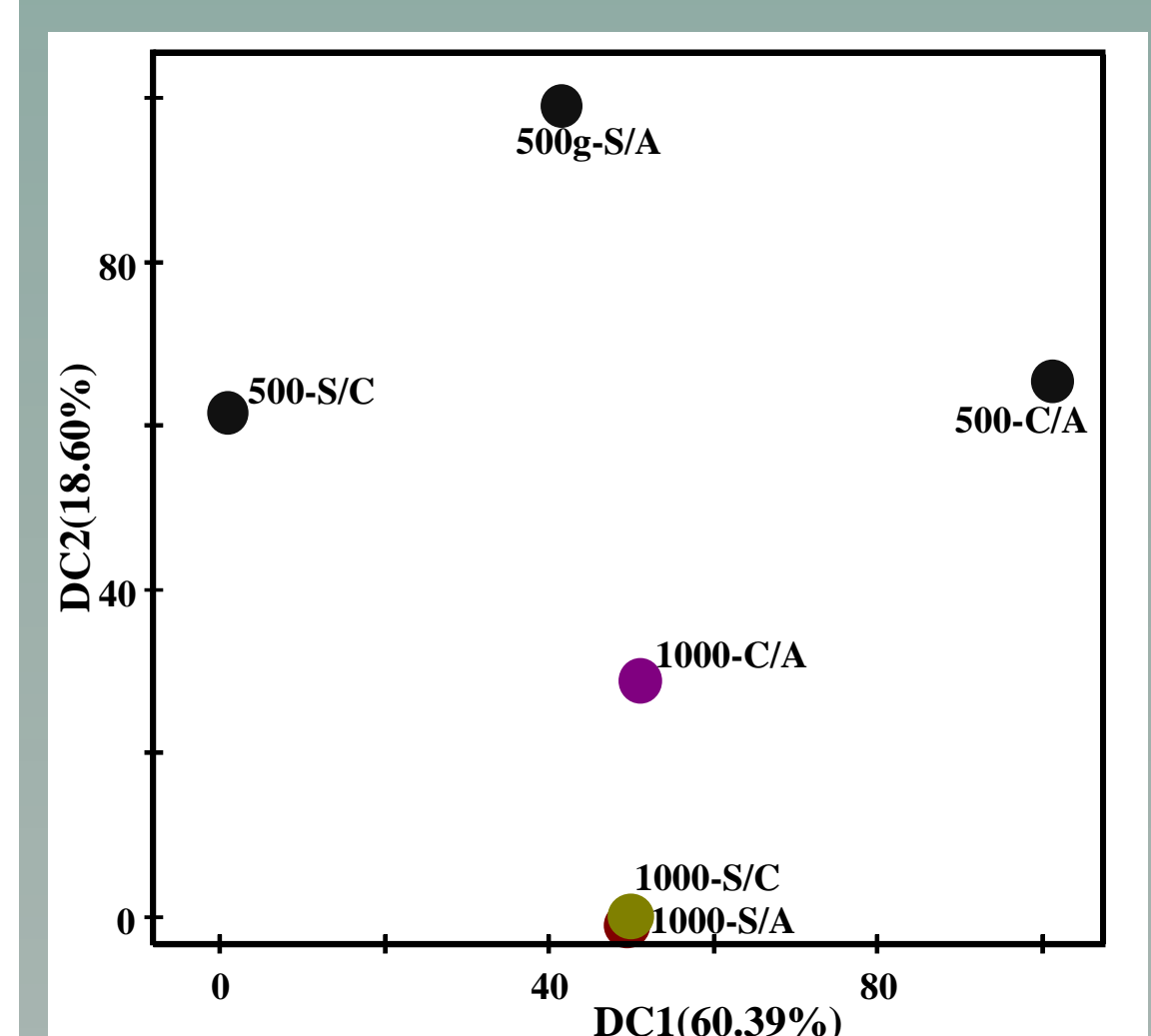
•ANOVA data are shown. Pink: P<0.001; light cyan, P<0.005).  
•Left: 1200 generation lines vs Ancestor. The salt-adapted lines performed better than the control lines in yield and growth rate, suggesting that they have acquired specific mutations that enable better growth on salt. Adaptation to LS4D without salt also provides some level of resistance to high salt levels---incidental improvements in salt tolerance that arise from pleiotropy. Maximal adaptation to salt may come at a cost to fitness in the absence of salt.  
•Right: Dynamics of the evolution. Evolved phenotype tend to stabilize at 1000 generations and mutational differences should occur before 1000 generations. The performance of salt-evolved lines in the control environment seemed to decline at 1200 generations---trade off.

### Changes of amino acid concentration in isolated evolved 1200g *D. vulgaris* clones

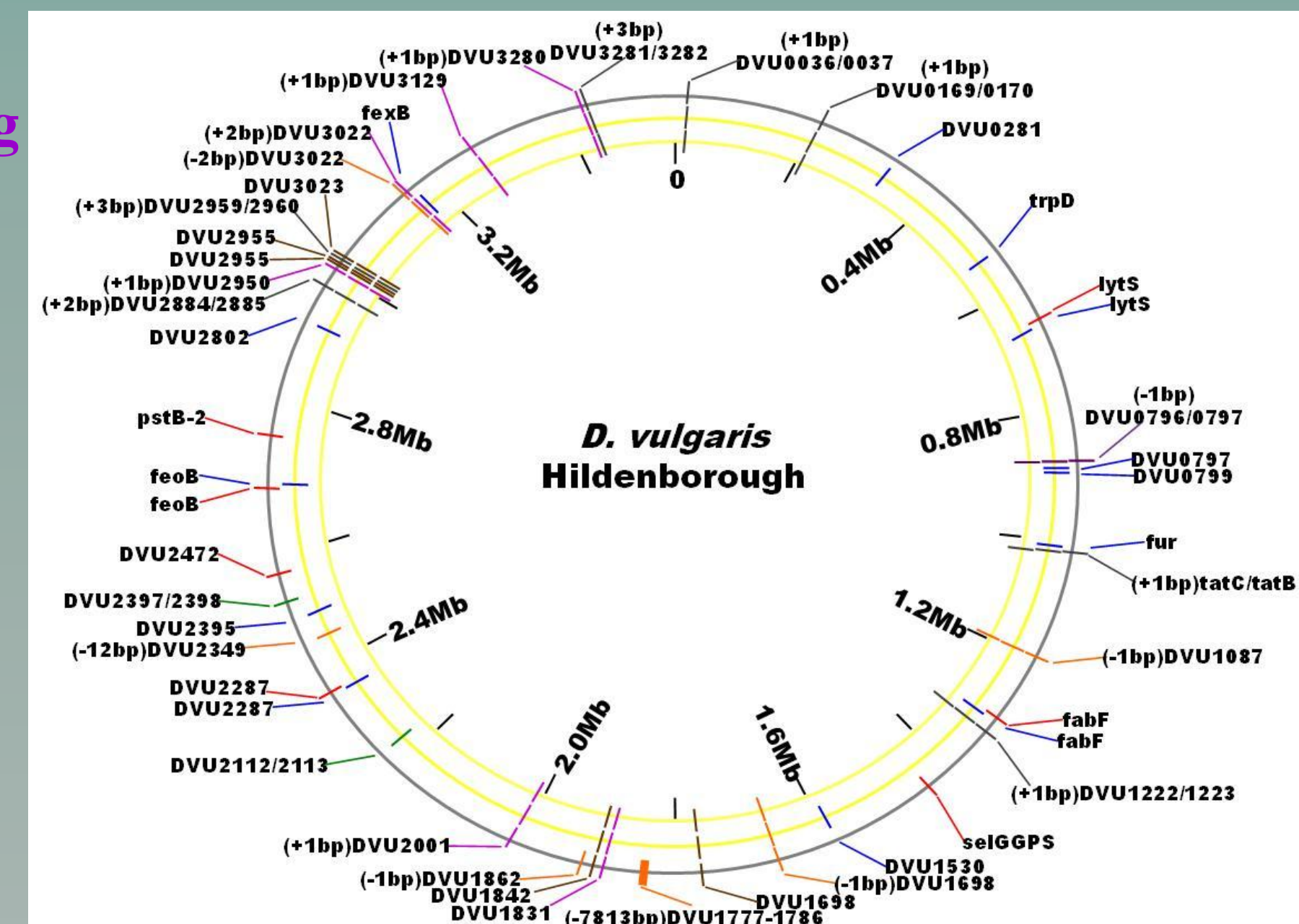
Metabolite	Summary of metabolites assay (pmoles/mg dry cell weight)									
	A - LS4D	A - LS4D	A - LS4D	C - LS4D	C - LS4D	C - LS4D	S - LS4D	S - LS4D	S - LS4D	S - LS4D
Name	0mM NaCl	100mM NaCl	250mM NaCl	0mM NaCl	100mM NaCl	250mM NaCl	0mM NaCl	100mM NaCl	250mM NaCl	0mM NaCl
Glycine	3.3711.35	3.7360.12	0.00	16.5211.65	14.8830.42	6.3421.47	24.1127.05	39.4665.55	21.1733.62	
Alanine	119.3216.22	184.9386.23	161.04237.82	390.82187.74	142.92111.45	98.84214.16	1991.33225.03	1891.13138.91	1848.962271.97	
Serine	0.4810.07	2.061.07	0.5220.28	1.3120.13	2.2120.21	0.2920.03	0.4920.04	1.8820.09	3.7621.97	
Proline	12.7810.09	17.7822.7	23.5422.04	16.111.17	13.3720.02	13.7121.03	13.5921.33	20.851.65	28.4221.32	
Valine	16.8822.87	27.98214.47	44.37211.1	116.16215.05	46.0328.9	27.311.76	84.6113	98.4628.07	116.96216.09	
Glycine betaine	7.851.29	10.1122.01	19.7420.11	9.1720.75	5.6120.17	5.4520.55	6.9820.15	28.320.66	8.3320.11	
Threonine	11.1320.9	25.5727.88	3664.61	51.4624.75	25.1124.63	21.2621.23	58.7429.18	66.9523.79	86.67210.39	
Aspartic acid	81.3219.5	129.84253.55	41.1720.05	216.86217.66	183.7224.39	112.1226.14	171.69214.25	277.83211.88	262.93219.82	
Ectoine	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	
Glutamine	53.93230.63	71.98228.46	51.5821.56	80.94213.53	61.2726.68	34.1625.55	94.37231.31	202.44236.12	124.33216.28	
Lysine	18.8220.39	21.3322.16	38.9421.07	22.8920.45	12.2921.22	13.3820.99	14.1621.25	17.9623.91	19.6322.57	
Glutamic acid	1183.69229.22	3727.312840.47	4222.87594.23	2988.44290.14	4410.212428.26	178.562268.53	1758.912175.05	5423.52252.6	8837.732742.68	
Methionine	4.8320.66	8.6623.08	8.8120.54	18.9821.06	9.0321.13	5.5520.38	14.9122.49	15.0220.64	17.1721.81	
Histidine	8.3320.3	9.9620.11	26.27232.8	9.2921.05	6.3520.25	5.5520.65	7.0320.94	6.6321.23	9.3727.59	
Phenylalanine	1.6420.31	1.3320.03	1.1320.38	8.7820.38	3.1920.24	1.0420.07	5.652.7	4.9820.69	3.8921.72	
Arginine	281.62	34.17.12	48.5120.95	42.1523.33	27.4520.77	20.8721.61	26.0422.63	33.4726.09	30.9123.47	
Tryptophan	0.5920.15	0.5320.05	0.00	1.6120.33	0.810.16	0.3920.05	1.6220.55	1.4420.13	0.6320.27	
Isoleucine	10.7321.51	10.6923.93	10.7820.88	27.3323.25	13.5422.72	7.211.79	25.6424.22	24.3327.24	21.822.39	
Leucine	15.7222.09	16.3426.93	18.9424.09	32.6522.76	17.1223.65	9.7720.67	37.5826.63	35.0427.13	34.3725.34	
Trehalose	0.0420.05	0.0520.03	0.1420.12	0.0420.04	0.0220.01	0.0520.05	0.0420.04	0.0320.01	0.0620.05	
Pyruvic acid	3.0120.3	3.0120.62	20.2122.81	5.220.72	2.0120.12	2.9520.42	4.8820.74	3.7620.31	4.9420.54	
α-Ketoglutaric acid	0.8120.64	0.5320.02	2.0620.34	0.7220.05	0.620.05	0.8920.1	0.4420.08	0.6520.03	1.6720.29	
Asparagine	0.5220.2	0.4820.28	0.8820.02	0.2920.06	0.1120.02	0.1920.04	2.0920.26	1.4720.44	1.1520.17	
Spermidine	40.84244.78	10.9320.55	10.2224.02	20.5923.21	8.4121.21	4.6921.45	9.6522.67	4.9322.12	10.7825.43	

Glu, Gln, Asp, Ala may be major osmoprotectants for relief of salt stress.

### The transcription profiling changes became stable at 1000g

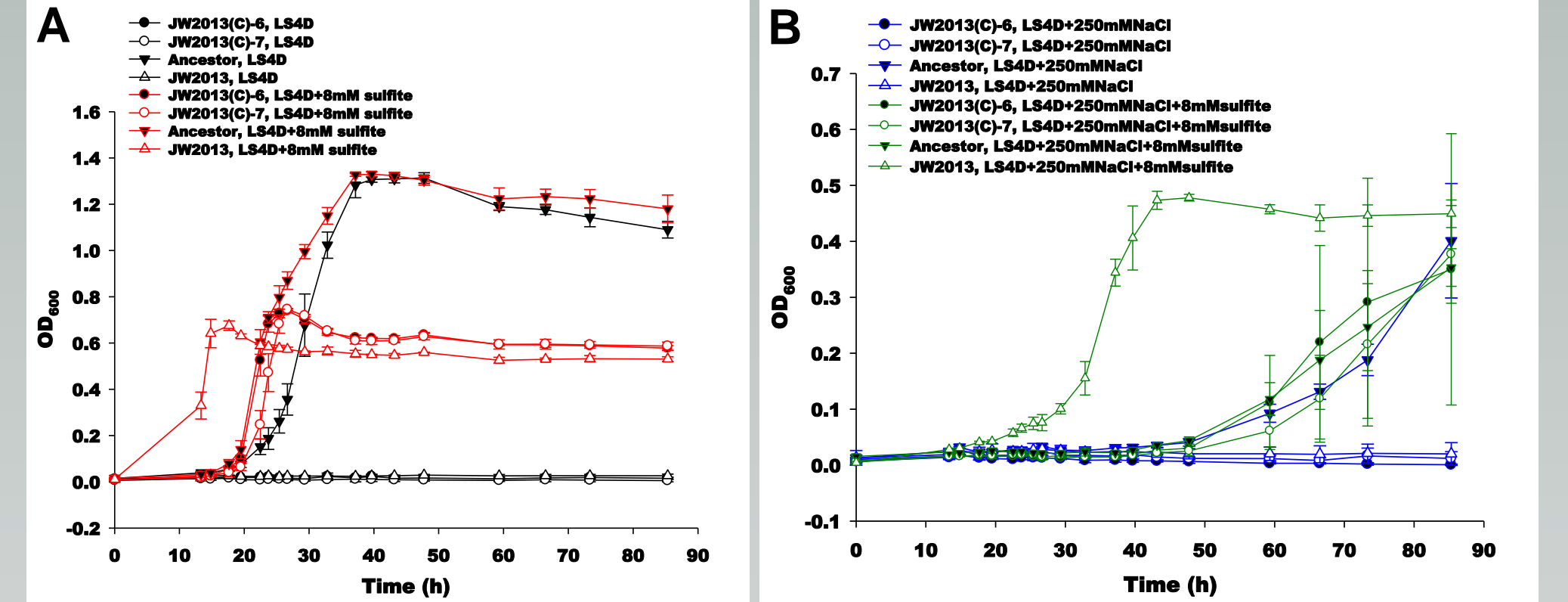


DCA analysis of the all the gene expression data was shown. S: evolved stressed lines; C: evolved control lines; A: ancestor



Mutations found by whole genome sequencing. Three rings (from inner to outer) represent ancestor, evolved control clone (3-10) and evolved salt stressed clone (9-11) sampled at 1200 generation. SNPs in coding region are labeled as red (9-11), blue (3-10), SNPs in intergenic region as green. Insertions in coding region are labeled as pink and intergenic region as dark grey. Deletions in coding region are labeled as orange and intergenic region as purple.

### The role of point mutation confirmed by complementation of knockout mutant



JW2013: knockout mutant of DVU0597; JW2013(c): complemented JW2013  
A: Sulfite is necessary for the growth of JW2013. Complementation of JW2013 partially rescued the growth phenotype of the mutant in the initiation of growth.  
B: With supplementation of sulfite, salt resistance of JW2013 was comparable to #9-11, salt resistance of complemented JW2013 was the same as wild type.

## SUMMARY

- *D. vulgaris* cell lines long-term transferred in control or salt stress condition adapted to their evolution environment;
- The salt-adapted lines acquired specific mutations that enable better growth on salt;
- A decrease of fitness of in the absence of salt suggested a trade-off in salt adaptation;
- Growth data and gene expression profiling analysis suggested that the evolved phenotype became stable at about 1000 generations;
- Point mutations were identified in evolved control and treatment lines and most of the mutations were fixed or almost fixed;
- Point mutation in DVU0597 was a loss-of-function and the mutation contribute for the salt resistance.

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### ACKNOWLEDGEMENTS

ENIGMA is a Scientific Focus Area Program supported by the U. S. Department of Energy, Office of Science, Office of Biological and Environmental Research, Genomics:GTL Foundational Science through contract DE-AC02-05CH11231 between Lawrence Berkeley National Laboratory and the U. S. Department of Energy.